

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
13 November 2003 (13.11.2003)

PCT

(10) International Publication Number  
**WO 03/093469 A2**

(51) International Patent Classification<sup>7</sup>: **C12N 15/00**

(21) International Application Number: **PCT/CA03/00645**

(22) International Filing Date: 30 April 2003 (30.04.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
60/377,547 1 May 2002 (01.05.2002) US

(71) Applicant (for all designated States except US): **CHROMOS MOLECULAR SYSTEMS, INC.** [CA/CA]; 8081 Lougheed Highway, Burnaby, British Columbia V5A 1W9 (CA).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **DE JONG, Gary** [CA/CA]; 656 Sylvan Avenue, North Vancouver, British Columbia V7R 2E9 (CA). **PERKINS, Edward** [US/CA]; 7610 Lawrence Drive, Burnaby, British Columbia V5A 1T6 (CA). **TELENIUS, Adele** [CA/CA]; 3869 Glengyle Street, Vancouver, British Columbia V5N 4S5 (CA). **MACDONALD, Neil** [CA/CA]; 7954 - 108th Street, Delta, British Columbia V4C 4C2 (CA).

(74) Agent: **SMART & BIGGAR**; Box 11560, Vancouver Centre, Suite 2200, 650 West Georgia Street, Vancouver, British Columbia V6B 4N8 (CA).

(81) Designated States (national): AE, AG, AL, AM, AT (utility model), AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ (utility model), CZ, DE (utility model), DE, DK (utility model), DK, DM, DZ, EC, EE (utility model), EE, ES, FI (utility model), FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK (utility model), SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— without international search report and to be republished upon receipt of that report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 03/093469 A2

(54) Title: METHODS FOR DELIVERING NUCLEIC ACID MOLECULES INTO CELLS AND ASSESSMENT THEREOF

(57) Abstract: Methods for delivering nucleic acid molecules into cells and methods for measuring nucleic acid delivery into cells and the expression of the nucleic acids are provided. The methods are designed for introduction of large nucleic acid molecules, including artificial chromosomes, into cells.

## METHODS FOR DELIVERING NUCLEIC ACID MOLECULES INTO CELLS AND ASSESSMENT THEREOF

### RELATED APPLICATIONS

- 5 This application is related to: U.S. application Serial No. 09/815,979 entitled "METHODS FOR DELIVERING NUCLEIC ACID MOLECULES INTO CELLS AND ASSESSMENT THEREOF", filed on March 22, 2001 by De Jong *et al.*; U.S. application Serial No. 09/815,981 entitled "METHODS FOR DELIVERING NUCLEIC ACID MOLECULES INTO CELLS AND ASSESSMENT THEREOF", filed on March 22, 2001
- 10 by De Jong *et al.*; U.S. application Serial No. 10/086,745 entitled "METHODS FOR DELIVERING NUCLEIC ACID MOLECULES INTO CELLS AND ASSESSMENT THEREOF", filed on February 28, 2002 by De Jong *et al.*; PCT International Application No. PCT/US02/09262 entitled "METHODS FOR DELIVERING NUCLEIC ACID MOLECULES INTO CELLS AND ASSESSMENT THEREOF", filed on March 22, 2002
- 15 by De Jong *et al.*; and, U.S. application Serial No. 60/377,547 entitled "METHODS FOR DELIVERING NUCLEIC ACID MOLECULES INTO CELLS AND ASSESSMENT THEREOF", filed on May 1, 2002 by De Jong *et al.*. The subject matter of each of these applications is incorporated by reference in its entirety.

### 20 FIELD OF THE INVENTION

The present invention relates to methods of delivering nucleic acid molecules into cells and methods for measuring nucleic acid delivery into cells and the expression of the nucleic acids therein.

### 25 BACKGROUND OF THE INVENTION

A number of methods for the delivery of nucleic acid molecules, particularly plasmid DNA and other small fragments of nucleic acid, into cells have been developed. Many of these methods are not ideal for delivery of larger nucleic acid molecules. Thus, there is a need for methods of delivering nucleic acid molecules of increasing size and complexity, 30 such as artificial chromosomes, into cells. Methods are required for use with *in vitro* and *in vivo* procedures such as gene therapy and for production of transgenic animals and plants. Furthermore, there is a need for the ability to determine and assess the efficiency of delivery of DNA into cells.

## SUMMARY OF THE INVENTION

In various aspects, the invention provides methods for delivering nucleic acid molecules, particularly larger molecules, including artificial chromosomes, into cells. Methods for assessing delivery are also provided. Selected methods may be used to deliver 5 nucleic acid molecules and/or proteins of a range of sizes, and may be particularly suitable for delivery of larger nucleic acid molecules, such as natural and artificial chromosomes with associated proteins and fragments thereof, into cells. Alternative methods may be designed for *in vitro* and *ex vivo* delivery of nucleic acid molecules for applications, including, but not limited to, delivery of nucleic acid molecules to cells for cell-based protein production, 10 transgenic protein production and gene therapy. Methods of protein production in cells and in transgenic animals and plants, methods of introducing nucleic acid into cells to produce transgenic animals and plants, and methods for *ex vivo* and gene therapy are also provided.

Methods provided herein are designed for delivering a large nucleic acid molecule with or without associated proteins into a cell, but may also be used to deliver smaller 15 molecules. Selected methods provided herein are designed for improving transfer of nucleic acid into the cells by delivering the nucleic acid into cells that are in a pre-selected phase. Cells of a pre-selected phase can be obtain in a number of ways. For example, cells can be isolated at a specific cell cycle stage using mechanical means such as elutriation or by exposing the cell to a cell cycle arrest agent or an agent that facilitates cell cycle 20 synchronization. The agent can be one or more compositions, conditions and/or physical treatments that facilitates cell arrest and/or results in a synchronized population of cells. Selected agents and combinations thereof can include; for example, those that result in the highest transfection efficiency, highest delivery efficiency and/or the greatest amount of 25 intact nucleic acid molecules transferred into the cell nucleus with an acceptable degree of cell survival. Thus, for example, the nucleic acid can be transferred to the cell at a phase or point in a phase of a cell that results in improved transfection efficiency, improved delivery efficiency and/or an improved proportion of intact nucleic acid molecules within the transfected cells (such improvements being relative to cells at a diffent phase or point in a phase of the cell cycle).

30 The selected methods may vary depending on the target cells (cells into which nucleic acid is delivered), the nucleic acid molecules, and methods used for obtaining cells in selected phases. Exemplary methods for delivery of large nucleic acid molecules into cells provided herein include obtaining a cell that is in a pre-selected phase of the cell cycle then

contacting the cell with a large nucleic acid molecule. The pre-selected phase of the cell may be determined by introducing a large nucleic acid into cells at different phases and determining and comparing the efficiency of delivery and/or of transfection of the nucleic acid into the cells at different phases and selecting a phase at which the efficiency of delivery and/or of transfection is increased relative to efficiency of delivery and/or transfection at other phases.

The pre-selected phase may be obtained by exposing one or more cells to a cell cycle arrest agent, exposing the cell(s) to conditions that permit cell cycling, and then selecting a cell that is in the pre-selected phase. Arrest agents may include, but are not limited to anti-microtubule agents such as nocodazole and agents that effect DNA replication such as thymidine. The pre-selected phase can be any phase including G0, G1, S, G2, M, or at the interface of two phases including G0/G1, G2/M, G1/S, and S/G2. In an exemplary method, the phase is one in which the nuclear membrane of the cell is absent.

The methods provided herein may be adapted for delivery of large nucleic acid molecules into cells in a variety of environments for a variety of purposes. For example, nucleic acid molecules greater than about 0.6, 1, and 5 megabase pairs may be delivered into cells using the methods provided herein.

Included among the nucleic acid molecules that may be delivered into cells using alternative embodiments of the methods provided herein are natural chromosomes, artificial chromosomes, artificial chromosome expression systems (ACes), or fragments of any of these chromosomes, such as fragments that are greater than about 0.6 megabase or naked DNA that is greater than about 0.6 megabase.

In particular embodiments of the methods a delivery agent may be used. Delivery agents may include a cationic compound. Cationic compounds include, but are not limited to, a cationic lipid, a cationic polymer, a mixture of cationic lipids, a mixture of cationic polymers, a mixture of a cationic lipid and a cationic polymer and a mixture of a cationic lipid and a neutral lipid, polycationic lipids, non-liposomal forming lipids, activated dendrimers, pyridinium chloride surfactants, ethanolic cationic lipids, and cationic amphiphiles. Examples of cationic lipid compounds include N-[1-(2,3-dioleyloxy)propyl]-30 N,N,N-trimethylammonium chloride (DOTMA), dioleoylphosphatidylethanolamine (DOPE), 2,3-dioleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate (DOSPA), C<sub>52</sub>H<sub>106</sub>N<sub>6</sub>O<sub>4</sub>.4CF<sub>3</sub>CO<sub>2</sub>H, C<sub>88</sub>H<sub>178</sub>N<sub>8</sub>O<sub>4</sub>S<sub>2</sub>.4CF<sub>3</sub>CO<sub>2</sub>H, C<sub>40</sub>H<sub>84</sub>NO<sub>3</sub>P.CF<sub>3</sub>CO<sub>2</sub>H, C<sub>50</sub>H<sub>103</sub>N<sub>7</sub>O<sub>3</sub>.4CF<sub>3</sub>CO<sub>2</sub>H,

C<sub>55</sub>H<sub>116</sub>N<sub>8</sub>O<sub>2</sub>.6CF<sub>3</sub>CO<sub>2</sub>H, C<sub>49</sub>H<sub>102</sub>N<sub>6</sub>O<sub>3</sub>.4CF<sub>3</sub>CO<sub>2</sub>H, C<sub>44</sub>H<sub>89</sub>N<sub>5</sub>O<sub>3</sub>.2CF<sub>3</sub>CO<sub>2</sub>H,  
C<sub>100</sub>H<sub>206</sub>N<sub>12</sub>O<sub>4</sub>S<sub>2</sub>.8CF<sub>3</sub>CO<sub>2</sub>H, C<sub>41</sub>H<sub>78</sub>NO<sub>8</sub>P) C<sub>162</sub>H<sub>330</sub>N<sub>22</sub>O<sub>9</sub>.13CF<sub>3</sub>CO<sub>2</sub>H,  
C<sub>43</sub>H<sub>88</sub>N<sub>4</sub>O<sub>2</sub>.2CF<sub>3</sub>CO<sub>2</sub>H, C<sub>43</sub>H<sub>88</sub>N<sub>4</sub>O<sub>3</sub>.2CF<sub>3</sub>CO<sub>2</sub>H, and (1-methyl-4-(1-octadec-9-enyl-  
nonadec-10-enylenyl) pyridinium chloride.

5 In particular embodiments of the methods the nucleic acid molecule can be exposed to an agent that increases contact between the nucleic acid molecule and the cell and the cell may be exposed to an agent that enhances permeability of the cell. The permeability enhancing agent may include energy such as ultrasound or electrical energy.

In alternative embodiments the invention may be utilized to introduce nucleic acids  
10 into a wide range of cell types, eukaryotic and prokaryotic, including cell lines, primary cells, primary cell lines, plant cells, and animal cells, including embryonic cells, nuclear transfer donor cells, stem cells, primary cells, immortalized cells, cells from immortalized cell lines, tumor cells, transformed cells, and cells that are capable of the generation of a specific organ.  
15 Animal cells may include mammalian cells, rodent cells or human cells. Exemplary cells for use in the methods provided herein include fibroblasts, synoviocytes, and fibroblast-like synoviocyte.

## **DETAILED DESCRIPTION OF THE INVENTION**

### **A. DEFINITIONS**

20 Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which this invention belongs. All patents, patent applications and publications referred to herein are incorporated by reference.

As used herein, "nucleic acid" refers to a polynucleotide containing at least two  
25 covalently linked nucleotide or nucleotide analog subunits. A nucleic acid can be a deoxyribonucleic acid (DNA), a ribonucleic acid (RNA), or an analog of DNA or RNA. Nucleotide analogs are commercially available and methods of preparing polynucleotides containing such nucleotide analogs are known (Lin *et al.* (1994) *Nucl. Acids Res.* 22:5220-5234; Jellinek *et al.* (1995) *Biochemistry* 34:11363-11372; Pagratis *et al.* (1997) *Nature Biotechnol.* 15:68-73). The nucleic acid can be single-stranded, double-stranded, or a mixture thereof. For purposes herein, unless specified otherwise, the nucleic acid is double-stranded, or it is apparent from the context.

The term “nucleic acid” refers to single-stranded and/or double-stranded polynucleotides, such as deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), as well as analogs or derivatives of either RNA or DNA. Also included in the term **nucleic acid** are analogs of nucleic acids such as peptide nucleic acid (PNA), phosphorothioate DNA, and other such analogs and derivatives.

5 As used herein, DNA is meant to include all types and sizes of DNA molecules including cDNA, plasmids and DNA including modified nucleotides and nucleotide analogs.

As used herein, nucleotides include nucleoside mono-, di-, and triphosphates.

Nucleotides also include modified nucleotides, such as, but are not limited to,  
10 phosphorothioate nucleotides and deazapurine nucleotides and other nucleotide analogs.

As used herein, the term “large nucleic acid molecules” or “large nucleic acids” refers to a nucleic acid molecule of at least about 0.5 megabase pairs (Mbase) in size, greater than about 0.5 Mbase, including nucleic acid molecules at least about 0.6, 0.7, 0.8, 0.9, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 87, 90, and 100, 200, 300, or 500 Mbase in size. Large  
15 nucleic acid molecules can include proteins associated with the molecule. Large nucleic acid molecules typically may be on the order of about 10 to about 450 or more Mbase, and may be of various sizes, such as, for example, from about 250 to about 400 Mbase, about 150 to about 200 Mbase, about 90 to about 120 Mbase, about 60 to about 100 Mbase and about 15 to 50 Mbase.

20 Examples of large nucleic acid molecules include, but are not limited to, natural chromosomes and fragments thereof, especially mammalian chromosomes and fragments thereof which retain a centromere and telomeres, artificial chromosome expression systems (ACes; also called satellite DNA-based artificial chromosomes (SATAcs); see U.S. Patent Nos. 6,025,155 and 6,077,697), mammalian artificial chromosomes (MACs), plant artificial  
25 chromosomes, insect artificial chromosomes, avian artificial chromosomes and minichromosomes (see, e.g., U.S. Patent Nos. 5,712,134, 5,891,691 and 5,288,625). The large nucleic acid molecules may include a single copy of a desired nucleic acid fragment encoding a particular nucleotide sequence, such as a gene of interest, or may carry multiple copies thereof or multiple genes or different heterologous sequences of nucleotides. For  
30 example, ACes can carry 40 or even more copies of a gene of interest. Large nucleic acid molecules may be associated with proteins. The protein can be any protein, including but not limited to, for example, chromosomal proteins, such as those that typically function to regulate gene expression and/or participate in determining overall structure and/or proteins

that are not typically associated with chromosomes in a cell. Examples of a protein not typically associated with a chromosome include, but are not limited to, recombinant proteins that have been combined with a nucleic acid, for example a large nucleic acid, within or outside of a cell.

5 As used herein, a “functional fragment” of a chromosome refers to a fragment of a chromosome that retains the basic functional attributes of a chromosome. Thus, for example, a functional fragment of a chromosome can stably replicate and segregate in a cell in a manner similar to a chromosome from which the fragment is derived. A functional fragment of a chromosome can be generated in a number of ways. For example, a functional fragment 10 can be generated by treating a complete, intact chromosome in such a way as to delete a portion or portions of the nucleic acid of the chromosome that are not required for the basic functioning of the chromosome in a cell.

As used herein, an artificial chromosome is a nucleic acid molecule that can stably replicate and segregate alongside endogenous chromosomes in a cell. It has the capacity to 15 act as a gene delivery vehicle by accommodating and expressing foreign genes contained therein. A mammalian artificial chromosome (MAC) refers to chromosomes that have an active mammalian centromere(s). Plant artificial chromosomes, insect artificial chromosomes and avian artificial chromosomes refer to chromosomes that include plant, insect and avian centromeres, respectively. A human artificial chromosome (HAC) refers to 20 chromosomes that include human centromeres. For exemplary artificial chromosomes, see, e.g., U.S. Patent Nos. 6,025,155; 6,077,697; 5,288,625; 5,712,134; 5,695,967; 5,869,294; 5,891,691 and 5,721,118 and published International PCT application Nos, WO 97/40183 and WO 98/08964. An artificial chromosome can include proteins that are associated with the nucleic acid molecule.

25 As used herein, the term “satellite DNA-based artificial chromosome (SATAC)” is interchangeable with the term “artificial chromosome expression system (ACes)”. These artificial chromosomes are substantially all neutral non-coding sequences (heterochromatin) except for foreign heterologous, typically gene-encoding nucleic acid, that is interspersed within the heterochromatin for the expression therein (see U.S. Patent Nos. 6,025,155 and 6,077,697 and International PCT application No. WO 97/40183). Foreign genes contained in these artificial chromosome expression systems can include, but are not limited to, nucleic acid that encodes traceable marker proteins (reporter genes), such as fluorescent proteins, such as green, blue or red fluorescent proteins (GFP, BFP and RFP, respectively), other

reporter genes, such as  $\beta$ -galactosidase and proteins that confer drug resistance, such as a gene encoding hygromycin-resistance. Other examples of heterologous DNA include, but are not limited to, DNA that encodes therapeutically effective substances, such as anti-cancer agents, enzymes and hormones, and DNA that encodes other types of proteins, such as

5 antibodies.

As used herein, the terms "heterologous" and "foreign" with reference to nucleic acids, such as DNA and RNA, are used interchangeably and refer to nucleic acid that does not occur naturally as part of a genome or cell in which it is present or which is found in a location(s) and/or in amounts in a genome or cell that differ from the location(s) and/or 10 amounts in which it occurs in nature. It can be nucleic acid that is not endogenous to the cell and has been exogenously introduced into the cell. Examples of heterologous DNA include, but are not limited to, DNA that encodes a gene product or gene product(s) of interest introduced into cells, for example, for purposes of gene therapy, production of transgenic animals or for production of an encoded protein. Other examples of heterologous DNA 15 include, but are not limited to, DNA that encodes traceable marker proteins, such as a protein that confers drug resistance, DNA that encodes therapeutically effective substances, such as anti-cancer agents, enzymes and hormones, and DNA that encodes other types of proteins, such as antibodies.

As used herein, "delivery," which is used interchangeably with "transfection," refers 20 to the process by which exogenous nucleic acid molecules are transferred into a cell such that they are located inside the cell. Delivery of nucleic acids is a distinct process from expression of nucleic acids.

As used herein, "expression" refers to the process by which nucleic acid is translated 25 into peptides or is transcribed into RNA, which, for example, may be translated into peptides, polypeptides or proteins. If the nucleic acid is derived from genomic DNA, expression may, if an appropriate eukaryotic host cell or organism is selected, include splicing of the mRNA. For heterologous nucleic acid to be expressed in a host cell, it must initially be delivered into the cell and then, once in the cell, ultimately reside in the nucleus.

As used herein, cell recovery refers to a "total cell yield" after a specified time frame, 30 which for purposes herein is twenty-four hours, and when used with reference to calculation of the clonal fraction.

As used herein, cell recovery time refers to a time frame in order for a cell to equilibrate to new conditions.

As used herein, cell survival refers to cell viability after a cytotoxic event, such as a delivery procedure.

As used herein, control plating efficiency (CPE) refers to the fraction of untreated cells, under standard optimal growth conditions for the particular cells, that survive a plating procedure. Plating efficiency refers to the fraction of treated cells that survive a plating procedure.

As used herein, clonal fraction is a measurement of cell recovery after delivery of exogenous nucleic acids into cells and the plating efficiency of the cells.

As used herein, transfer or delivery efficiency is the percentage of the total number of cells to which nucleic acids are delivered that contain delivered nucleic acid.

As used herein, transfection efficiency is the percentage of the total number of cells to which nucleic acids including a selectable marker are delivered that survive selection and/or express the selectable marker.

As used herein, index of potential transfection efficiency means the theoretical maximum transfection efficiency for a particular cell type under particular conditions, for example particular concentrations or amounts of particular delivery agents.

As used herein, the term "cell" is meant to include cells of all types, of eukaryotes and prokaryotes, including animals and plants.

As used herein, the term "cell cycle" includes the reproductive cycles through which a cell grows and divides to yield two or more daughter cells of the same or different ploidy. The cell cycle can be viewed as having several distinct phases, including, for example, in a eukaryotic organism, the G<sub>1</sub>, S, G<sub>2</sub> and M phases. The M phase is the mitotic phase which includes segregation of the pairs of chromosomes, the production of two separate nuclei and division of the cell into two daughter cells. The M phase can be further divided into prophase, prometaphase, metaphase, anaphase, telophase and cytokinesis. In meiosis, similar phases occur in both the first and second cell divisions that typically yield four daughter cells. Typically, the G<sub>1</sub>, S and G<sub>2</sub> phases are referred to as forming a period known as interphase during which the cell grows and progresses toward mitosis. During the S phase, DNA synthesis can occur in preparation for cell division.

As used herein, "phase" with reference to a cell refers to a state of a cell relative to the cell cycle. Thus, for example, the phase of a cell can be a phase within the active cell cycle (e.g., G<sub>1</sub>, S, G<sub>2</sub> and M) or a phase out of the cell cycle, such as the resting phase referred to as G<sub>0</sub> in which the cell is quiescent and not actively passing through the cell cycle.

As used herein, "cell cycle arrest agent" refers to any means chemical, physical or others used to stop a cell from passing a set point or points in the growth cycle of a cell.

As used herein, "pre-selected phase" refers to a phase or point within a phase of a cell that specifically has been selected. The selection of a particular phase or point within a phase 5 of a cell can be based on a determination of a characteristic of that point or phase. Thus, for example, the selection of a phase or point within a phase of a cell may be based on a determination that cells at that point or phase provide for improved transfer of a nucleic acid, particularly a large nucleic acid, into the cells relative to the transfer of the nucleic acid into cells at a different point or phase. Improved transfer can be, for example, any qualitative 10 and/or quantitative improvement in any aspect of process and/or result of the transfer of the nucleic acid. Thus, improved transfer can be, for example, an increase in the efficiency of delivery and/or transfection of the nucleic acid into the cell, an increase in expression of the delivered nucleic acid into the cell and/or an increase in the integrity or intactness of the delivered nucleic acid in the cell.

15 As used herein, "conditions that permit cell cycling" refers to conditions that allow for growth of cells.

As used herein, a "synchronous population" with reference to a population of cells is one in which more cells in the population are in the same single phase than in an equivalent 20 population of cells that has not been synchronized. Synchronization is effected by exposing a population of cells to conditions that result in an increase in the number of cells in the population that are in the same single phase. Such conditions are known by those of skill in the art and include but are not limited to natural and artificial treatments, such as, for example, changing any internal or external parameter of the cells, arresting cycling of the 25 cells and releasing the cells from arrest, and physical separation of cells that are in different phases. An asynchronous population of cells is one which has not been exposed to any conditions that render the cells synchronous.

As used herein, "delivery agent" refers to compositions, conditions or physical treatments to which cells and/or nucleic acids may be exposed in the process of transferring nucleic acids to cells in order to facilitate nucleic acid delivery into cells. Delivery agents 30 include compositions, conditions and physical treatments that enhance contact of nucleic acids with cells and/or increase the permeability of cells to nucleic acids. In general, nucleic acids are not directly treated with energy, such as sonoporation.

As used herein, cationic compounds are compounds that have polar groups that are positively charged at or around physiological pH. These compounds facilitate delivery of nucleic acid molecules into cells; it is thought this is achieved by virtue of their ability to neutralize the electrical charge of nucleic acids. Exemplary cationic compounds include, but 5 are not limited to, cationic lipids or cationic polymers or mixtures thereof, with or without neutral lipids, polycationic lipids, non-liposomal forming lipids, ethanolic cationic lipids and cationic amphiphiles. Contemplated cationic compounds also include activated dendrimers, which are spherical cationic polyamidoamine polymers with a defined spherical architecture of charged amino groups which branch from a central core and which can interact with the 10 negatively charged phosphate groups of nucleic acids (e.g., starburst dendrimers).

Cationic compounds for use as delivery agents also include mixtures of cationic compounds that include peptides and protein fragments. The additional components may be non-covalently or covalently bound to the cationic compound or otherwise associated with the cationic compound.

15 As used herein, ultrasound energy is meant to include sound waves (for external application) and lithotripter-generated shock waves (for internal application).

As used herein, electrical energy is meant to include the application of electric fields to cells so as to open pores in membranes for the delivery of molecules into the cell, e.g., electroporation techniques.

20 As used herein, cavitation compound is meant to include contrast agents that are typically used with ultrasound imaging devices and includes gas encapsulated and nongaseous agents. These cavitation compounds enhance the efficiency of energy delivery of acoustic or shock waves.

25 As used herein, "pharmaceutically acceptable" refers to compounds, compositions and dosage forms that are suitable for administration to the subject without causing excessive toxicity, irritation, allergic response or other undesirable complication.

As used herein, embryonic stem cells are primitive, immature cells that are precursors to stem cells.

30 As used herein, stem cells are primitive, immature cells that are precursors to mature, tissue specific cells.

As used herein, nuclear transfer donor cells are cells that are the source of nuclei, which are transferred to enucleated oocytes during the process of nuclear transfer.

As used herein, the term "subject" refers to animals, plants, insects, and birds into which the large DNA molecules may be introduced. Included are higher organisms, such as mammals and birds, including humans, primates, rodents, cattle, pigs, rabbits, goats, sheep, mice, rats, guinea pigs, cats, dogs, horses, chicken and others.

5 As used herein, "administering to a subject" is a procedure by which one or more delivery agents and/or large nucleic acid molecules, together or separately, are introduced into or applied onto a subject such that target cells which are present in the subject are eventually contacted with the agent and/or the large nucleic acid molecules.

10 As used herein, "applying to a subject" is a procedure by which target cells present in the subject are eventually contacted with energy such as ultrasound or electrical energy. Application is by any process by which energy may be applied.

15 As used herein, gene therapy involves the transfer or insertion of nucleic acid molecules, and, in particular, large nucleic acid molecules, into certain cells, which are also referred to as target cells, to produce specific gene products that are involved in correcting or modulating diseases or disorders. The nucleic acid is introduced into the selected target cells in a manner such that the nucleic acid is expressed and a product encoded thereby is produced. Alternatively, the nucleic acid may in some manner mediate expression of DNA that encodes a therapeutic product. This product may be a therapeutic compound, which is produced in therapeutically effective amounts or at a therapeutically useful time. It may also 20 encode a product, such as a peptide or RNA, that in some manner mediates, directly or indirectly, expression of a therapeutic product. Expression of the nucleic acid by the target cells within an organism afflicted with a disease or disorder thereby provides a way to modulate the disease or disorder. The nucleic acid encoding the therapeutic product may be modified prior to introduction into the cells of the afflicted host in order to enhance or 25 otherwise alter the product or expression thereof.

For use in gene therapy, cells can be transfected *in vitro*, followed by introduction of the transfected cells into the body of a subject. This is often referred to as *ex vivo* gene therapy. Alternatively, the cells can be transfected directly *in vivo* within the body of a subject.

30 As used herein, flow cytometry refers to processes that use a laser based instrument capable of analyzing and sorting out cells and or chromosomes based on size and fluorescence.

As used herein, a reporter gene includes any gene that expresses a detectable gene product, which may be RNA or protein. Preferred reporter genes are those that are readily detectable. Examples of reporter genes include, but are not limited to nucleic acid encoding a fluorescent protein, CAT (chloramphenicol acetyl transferase) (Alton and Vapnek (1979), 5 *Nature* 282: 864-869) luciferase, and other enzyme detection systems, such as beta-galactosidase; firefly luciferase (deWet *et al.* (1987), *Mol. Cell. Biol.* 7: 725-737); bacterial luciferase (Engebrecht and Silverman (1984), *PNAS* 1: 4154-4158; Baldwin *et al.* (1984), *Biochemistry* 23: 3663-3667); and alkaline phosphatase (Toh *et al.* (1989) *Eur. J. Biochem.* 182: 231-238, Hall *et al.* (1983) *J. Mol. Appl. Gen.* 2: 101).

As used herein, a reporter gene construct is a DNA molecule that includes a reporter gene operatively linked to a transcriptional control sequence. The transcriptional control sequences include a promoter and other optional regulatory regions, such as enhancer sequences, that modulate the activity of the promoter, or control sequences that modulate the activity or efficiency of the RNA polymerase that recognizes the promoter, or control 10 sequences that are recognized by effector molecules, including those that are specifically induced by interaction of an extracellular signal with a cell surface protein. For example, modulation of the activity of the promoter may be effected by altering the RNA polymerase binding to the promoter region, or, alternatively, by interfering with initiation of transcription or elongation of the mRNA. Such sequences are herein collectively referred to as 15 transcriptional control elements or sequences. In addition, the construct can include sequences of nucleotides that alter translation of the resulting mRNA, thereby altering the amount of reporter gene product.

As used herein, promoter refers to the region of DNA that is upstream with respect to the direction of transcription of the transcription initiation site. It includes the RNA 20 polymerase binding and transcription initiation sites and any other regions, including, but not limited to repressor or activator protein binding sites, calcium or cAMP responsive sites, and any such sequences of nucleotides known to those of skill in the art to alter the amount of transcription from the promoter, either directly or indirectly.

As used herein, a promoter that is regulated or mediated by the activity of a cell 25 surface protein is a promoter whose activity changes when a cell is exposed to a particular extracellular signal by virtue of the presence of cell surface proteins whose activities are affected by the extracellular protein.

**B. CELL CYCLE****1. Cell Cycle Phases**

In the reproductive cycle of a cell, the cell undergoes specific phases of DNA synthesis and division over a defined period of time. In a somatic mammalian cell, this time course is conventionally divided into four phases: G1 (first gap) phase, S (DNA synthesis) phase, G2 (second gap) phase, and M (mitotic) phase. Ninety percent or more of the total cell-cycle time course is dominated by the G1, S and G2 phases, collectively termed interphase. The remaining time is devoted to the M phase, during which the cell actually divides. For example, in rapidly proliferating cells of higher eukaryotes, M phases generally occur only once every 16 to 24 hours, and each M phase itself lasts only 1 to 2 hours. Cells in different tissues, in different species, and at different stages of embryonic development have division cycles that vary greatly in duration, from less than an hour (for example, in the early frog embryo) to more than a year (for example, in the adult human liver). Although all phases of the cell cycle vary to some extent, by far the greatest variation occurs in the duration of G1, which may be, in some cases, practically non-existent, or for such a length of time that the cell appears to have altogether ceased progressing through the divisional cycle and to have withdrawn into a quiescent state. Cells in such a quiescent G1 state are often said to be in the G0 state.

During the M phase or mitosis, the phase of cell division, the cell transitions through four substages: prophase, metaphase, anaphase, and telophase. In meiosis, similar substages occur in each of the two cellular division cycles that typically yield four daughter cells. Prophase involves the condensation of chromosomes and breakdown of nuclear membrane such that the nuclear contents are no longer separated from the cytoplasm. Metaphase is characterized by the migration of chromosomes to the equatorial plane of the cell. In anaphase, the chromosomes migrate to opposite poles of the cell segregating half sets of chromosomes to the two ends of the dividing cell. In late anaphase, constriction through the equator of the cell begins and results in the pinching off of two daughter cells in telophase. The daughter cells then begin interphase of a new cycle.

Meiosis typically comprises two successive nuclear divisions with only one round of DNA replication. Four stages can be described for each nuclear division, similar to the stages of mitosis. The first division of meiosis may be divided into the following substages: prophase 1, when each chromosome duplicates and remains closely associated (called sister chromatids); metaphase 1, when homologous chromosomes align at the equatorial plate;

anaphase 1, when homologous pairs separate with sister chromatids remaining together; and, telophase 1, when two daughter cells are formed with each daughter containing only one chromosome of the homologous pair. Similarly, The second division of meiosis may be divided into the following substages: prophase 2, when DNA does not replicate; metaphase 2, 5 when chromosomes align at the equatorial plate; anaphase 2, when centromeres divide and sister chromatids migrate separately to each pole; and, telophase 2, when cell division is complete. The following detailed discussion relates largely to the mechanics of mitotic division, although it applies *mutatis mutandis* to cells undergoing meiosis.

Interphase starts with the G1 phase, in which the biosynthetic activities of the cells, 10 which proceed very slowly during mitosis, resume at a high rate. During G1, the cell is subject to stimulation by extracellular mitogens and growth factors. In response to these stimuli, the cell passes through G1 and proceeds with DNA synthesis in S phase. The S phase begins when DNA synthesis starts, and ends when the DNA content of the nucleus has doubled and the chromosomes have replicated. The cell then enters the G2 phase, which 15 continues until mitosis starts, initiating the M phase.

## 2. Cell Cycle Control

The regulation of the cell-cycle must ensure that the events in each phase are complete before moving to the next. To ensure completion of each phase, checkpoints for monitoring the integrity of DNA are strategically placed in G1 and at the G2/M interface to 20 prevent progression and propagation of mutated or damaged cells. The G1 checkpoint can be described as the transition from early G1, when cells have the option of withdrawing from the cell cycle into the G0 state, and late G1, when cells are committed to another round of genome replication and division. In mammalian cells, this transition is also referred to as the “restriction point”. In response to growth and/or mitotic signals, the cell traverses the 25 restriction point and commits to proceeding through the cell cycle. In the absence of such signals, the cell may undergo differentiation, apoptosis, or enter the quiescent state (G0). Once committed, the cell cycle is propelled by a series of cyclin/cyclin dependent protein kinase (CDK) complexes. Thus, the transition between cell cycle stages is delineated by the synthesis and subsequent proteolytic degradation of various cyclins.

30 For example, the mammalian cycle begins in G1 with increased expression of the D cyclins (D1, D2, D3). D cyclin activity has been linked to cell size and external growth-regulatory signals coupling cell size with entry into a new cell cycle. The D cyclins associate with CDK4 and CDK6 resulting in phosphorylation and activation of the CDKs. Activated

CDK4/6 mediates a signal cascade resulting in the phosphorylation of the retinoblastoma protein and subsequent release of E2F transcription proteins and transcription of responder genes (including cyclin E). The responder proteins are required for G1 progression through the restriction point.

5 As the cell progresses through late G1, Cyclin E/CDK2 facilitates processes associated with the transition from G1 into S, primarily by shutting down the various braking systems that suppress S phase CDK activity in G1. Cyclin E/CDK2 also initiates other early cell-cycle events, such as the duplication of the centrosome. Increased expression of cyclin A begins at the G1/S transition and continues through S phase. Cyclin A binding to CDK2  
10 stimulates DNA synthesis. In late S phase cyclin A binds CDK1. As the cell approaches M phase, cyclin B expression increases and peaks at metaphase. Cyclin B/CDK1 along with Cyclin A/CDK1 activity propels the cell through mitosis. The degradation of these cyclins result in exit from mitosis and cytokinesis.

### 3. Chromosome Replication and Control

15 The synthesis of genomic DNA in S phase is only one component of the chromosomal replication process. A number of important processes throughout the cell cycle and specifically cell division are coupled to the DNA replication process and the proteins and factors which drive this process. In addition, many of the proteins and factors that are crucial for progression through the various cell cycle phases may be useful targets for cell arrest  
20 agents.

In eukaryotic cells, the DNA molecules of the chromosomes begin replication or synthesis at multiple start points or replication origins distributed along the individual chromosomes. Synthesis begins bidirectionally at these points and proceeds until the replication fork of adjacent start points meet. Although replication origins can vary greatly  
25 depending on cell type, they can be determined, in part, by the binding of the origin recognition complex (ORC), which has been conserved in evolution from yeast to humans. In most cells, one or more subunits of the ORC remain bound to origins through most of the cell cycle. For example, in multicellular eukaryotes, some ORC subunits remain bound throughout G1, S and G2 phases. Early in the G1 phase of the cell cycle, proteins required  
30 for DNA synthesis, including Cdc6, Cdt1 and six related proteins known as the MCM2-7 complex, are recruited to the ORC to form the prereplicative complex (pre-RC). By blocking the recruitment of such proteins, the cell can be arrested in G1/G0 phase. In late G1 phase, the pre-RC matures into a pre-initiation complex (pre-IC) by recruitment of additional factors

including Cdc45, Sld3 and other factors involved in initiation and elongation during DNA synthesis.

At the end of G1, when the initiation complex at each replication origin is complete, a specific cyclin\CDK complex triggers the initiation of DNA synthesis and the S phase. In 5 mammalian cells, this complex is cyclin A/CDK2. Another essential S phase promoting kinase is Cdc7 which interacts directly with the replication origin and/or ORC complex. After CDK and Cdc7 activation, origins are unwound and the heterotrimeric single-stranded DNA binding protein RPA is recruited. After early unwinding in early S phase, DNA polymerase  $\alpha$ -primase complex is recruited to facilitate early synthesis. After initiation of 10 early synthesis, additional DNA polymerases and auxiliary factors such as PCNA (proliferating cell nuclear antigen) are recruited to complete synthesis of each parental strand. As chromosomal DNA unwinds during synthesis, the topology of the DNA changes. Unwinding generates positive supercoils ahead of the replication fork and precatenanes in the newly replicated DNA behind the fork. The topology continues to change through mitosis. 15 Agents which block the action or recruitment of required DNA synthesis machinery, such as, for example, DNA polymerase, ribonucleotide reductase or PCNA, will arrest the cell in S phase. PCNA also plays a central role in coupling many other cell cycle processes to the replication fork via direct protein protein interactions such as chromatid cohesion in late S phase and/or G2 phase. DNA damage and/or stalled replication forks trigger a global 20 genome integrity checkpoint and recruitment of a number of kinases and other factors. Activation of this checkpoint prevents the firing of additional replication origins and prevents entry into mitosis, thus agents that target this checkpoint may be used to arrest cells in S and/or G2 phase.

In G2 phase, after DNA synthesis, the two daughter DNA molecules remain tightly 25 associated with each other in a process known as sister chromatid cohesion. Cohesion is believed to occur discontinuously across the chromatids at specific and numerous cohesion sites, including centromeres. In the G2 phase, cohesion may help facilitate post-replicative repair of any double stranded breaks. Cohesion is also important for events occurring in M phase. For example, after assembly of a bipolar mitotic spindle in metaphase, controlled 30 proteolysis of cohesions allows the two sister chromatids to be pulled to opposite poles and initiate anaphase. Attachment of the chromatids to microtubules (which form the mitotic spindle) from opposite poles by kinetochores is a crucial step in the segregation process and, therefore, factors which facilitate this process are useful targets for M phase cell arrest agents.

Chromosome segregation coincides with cyclin B degradation which drives cytokinesis and mitotic exit.

### C. CELL PHASE-BASED DELIVERY OF NUCLEIC ACID INTO CELLS

Provided herein are methods of delivering nucleic acids into cells in which the

5 recipient cell is selected to be in a particular phase. The methods are particularly well suited for the delivery of large nucleic acids, including, for example, chromosomes and fragments thereof, and artificial chromosomes into cells. The methods utilize host cells that are in a phase that provide for transfer, and in particular improved transfer, of intact large nucleic acids into the cells. The phases or cycles of the cells in a host cell population that may be  
10 used in the methods can be synchronized to provide for an increased number of the cells that are in the same single phase for delivery of large nucleic acids into the cells. Methods for determining a cell phase for delivery of large nucleic acids into cells and methods for obtaining a cell or plurality of cells in a particular phase of the cell are also described herein.

#### 1. Cells

15 The methods provided herein can be used in the delivery of nucleic acids into any cells, including, but not limited to, any eukaryotic and prokaryotic cells. Examples of cells that can be used in the methods include, but are not limited to, cell lines, primary cells, primary cell lines, plant cells and animal cells, including stem cells and embryonic cells. For example, fibroblasts, including lung and skin fibroblasts, fibroblast-like cells, synoviocytes, 20 fibroblast-like synoviocytes, stem cells, including embryonic and adult stem cells, such as mesenchymal stem cells, myoblasts, lymphoblasts, carcinoma and hepatoma cells are among the many cells into which nucleic acids, and in particular large nucleic acids and artificial chromosomes, can be delivered and monitored using the methods provided herein. Particular cells include mammalian cells, for example, A9 cells (mouse fibroblasts, HPRT-; ATCC  
25 Accession no. CCL-1.4), CHO-S cells and DG44 cells (Chinese hamster ovary cells), V79 cells (Chinese hamster lung fibroblasts; ATCC Accession no. CCL-39), LMTK-cells (mouse fibroblasts; ATCC Accession No. CCL-1.3), skin fibroblasts (including primary human foreskin fibroblasts), L8 cells (rat myoblasts; ATCC Accession No. CRL-1769), CCD1043 SK cells (human fibroblasts; ATCC Accession No. CRL-2056), adult-derived mesenchymal 30 stem cells (e.g., derived from human bone marrow; Cambrex Biosciences, East Rutherford, New Jersey), synoviocytes (rat and human), Detroit 551 cells (human embryonic skin fibroblasts; ATCC Accession No. CCL-110), NS0 (murine myeloma, ECACC Accession No. 85110503), 293 cells (human embryonic kidney cells transformed by type 5 (Ad 5) DNA

(ATCC Accession No. CRL-1573), P46-FI (bovine lymphocyte-like cell line), DT40 (chicken lymphoblasts), EJ30 cells (human bladder carcinoma), HepG2 cells (human hepatoma) and murine and bovine embryos.

In particular embodiments, the methods of delivery of nucleic acids into cells

- 5 provided herein can be used in delivering nucleic acids into cells in order to treat a disease or disorder, e.g., in gene therapy applications. In gene therapy applications, the nucleic acid to be delivered into a cell may encode a therapeutic molecule, e.g., a protein. In a particular example, the protein may be Factor VIII. In many instances, successful gene therapy applications are complicated by a requirement that large nucleic acids be delivered into cells.
- 10 It may also be desired to provide multiple copies of nucleic acid encoding one or more therapeutic molecules. Compounding the difficulties in gene therapy methods is the challenge that cells preferred for use in gene therapy applications are often not readily transfectable. The methods provided herein are particularly well suited for delivery of large nucleic acids, which may be in the form of artificial chromosomes or fragments thereof, into
- 15 cells as may be used in therapeutic applications.

## 2. Nucleic acids

Although any nucleic acid may be used in the cell phase-based methods described herein, the methods are particularly well suited for delivery of large nucleic acids into cells. In particular embodiments, the large nucleic acids are chromosomes, fragments thereof and artificial chromosomes and any other large and/or complex nucleic acid. In further particular embodiments, the large nucleic acid is associated with one or more proteins through any type of binding or other interaction. The methods described herein provide for improved transfer of such nucleic acids into cells. For example, the cell phase-based methods described herein may provide for delivery of large nucleic acid molecules into cells with greater transfection efficiency and greater integrity of the molecules than methods that do not include use of a cell that is in a pre-selected phase. The efficiency of transfection of large nucleic acid molecules, and in particular chromosomes, into cells using these cell phase-based methods is at least 1.5, at least 2, at least 2.5, at least 3, at least 3.5, at least 4, at least 4.5, at least 5, at least 5.5, at least 6, at least 6.5, at least 7, at least 7.5, at least 8, at least 8.5, at least 9, at least 9.5 or at least 10-fold greater than the efficiency of transfection of the nucleic acid molecule to cells in an asynchronous population of cells.

In particular embodiments, the methods provide for the delivery of chromosomes, fragments thereof or artificial chromosomes into cells. Artificial chromosomes include

Artificial Chromosome Expression System (ACes; also called satellite DNA-based artificial chromosomes (SATACs)) chromosomes, which are described, for example, in U.S. Patent Nos. 6,025,155 and 6,077,697 and PCT International Patent Application Publication No. WO 97/40183, mammalian artificial chromosomes (MACs), plant artificial chromosomes, insect 5 artificial chromosomes, avian artificial chromosomes and minichromosomes (see, e.g., U.S. Patent Nos. 5,712,134, 5,891,691 and 5,288,625). For other exemplary artificial chromosomes, see, e.g., U.S. Patent Nos. 5,695,967; 5,869,294; and 5,721,118 and published International PCT application No. WO 98/08964.

In methods for delivering chromosomes, functional fragments thereof or artificial 10 chromosomes into cells as provided herein, the phase of the chromosome can be a consideration. During the cell cycle, chromosomes undergo morphological and other changes. For example, the topology of chromosomal DNA changes as it is unwound during DNA replication in the S phase of the cell cycle. Unwinding generates positive supercoils ahead of the replication fork and precatenanes in the newly replicated DNA behind the fork. 15 During mitosis, chromosomes undergo condensation and decondensation with accompanying changes in appearance detectable through microscopy. Typically, chromosomes begin to condense during prophase and to decondense during telophase of mitosis. Thus, the condition of a chromosome differs at different phases of the cell cycle.

The condition of a chromosome may be a consideration first in isolating a 20 chromosome from a cell and handling of the chromosome for subsequent transfer into a particular recipient cell. For example, condensation level may be a significant factor in the isolation and handling of chromosomes. Furthermore, the condition of a chromosome may impact its retention in a cell into which it is delivered. In particular methods provided herein for delivery of chromosomes or fragments thereof into cells, the phase of the chromosome or 25 fragment thereof is the same or similar to that of the recipient cell at the time of delivery. In further embodiments, the phase of the chromosome and of the recipient cell is the M phase, and, in particular, metaphase.

### 3. Determining cell cycle phase to be used in a method for delivering nucleic acid to a cell

30 In methods provided herein for delivering nucleic acids to cells, a recipient cell is in a pre-selected phase. The pre-selected phase can be one that can provide for improved transfer of a nucleic acid, such as a large nucleic acid, into the cell. The pre-selected phase can differ for different types of recipient cells and for different types of nucleic acids and can be

determined as described herein and using procedures known in the art. Improved transfer can take into account a number of factors. For example, delivery efficiency, transfection efficiency, integrity of the transferred molecules and cell viability after delivery are major considerations in determining improved transfer. Thus, for example, improved transfer may 5 be an increased delivery or transfection efficiency attainable while still retaining intact transferred nucleic acids in a certain percentage of the recipient cells.

**(a) Assessing cell phase and chromosome phase**

One way to determine the phase of the cell cycle that a recipient cell should be in for 10 delivery of the large nucleic acid into the cell involves determining and comparing the efficiencies of delivery and/or transfection, or comparing any other factor of relevance in achieving improved transfer, of nucleic acid into the cell in different phases of the cell cycle and selecting the cycle in which improved transfer of the nucleic acid is obtained. In order to compare the delivery and/or transfection efficiencies, or any other relevant parameter of transfer, the cell phases of the recipient cells are determined and the nucleic acid is contacted 15 with the cells of the different phases.

Methods of assessing the phase of a cell or population of cells are described herein and are known in the art [see, e.g., Darzynkiewicz *et al.* (2001) *Seminars Hematol.* 38:179-193 and Nunez (2001) *Curr. Issues Mol. Biol.* 3:67-70]. Cell phase may be assessed in a variety of ways including cytometric analysis, microscopic analysis, gradient centrifugation, 20 elutriation and fluorescence techniques including immunofluorescence (which may be used in combination with, for example, any of the preceding techniques). Cytometric techniques include exposing the cell to an agent or stain, such as DNA-binding dyes, e.g., propidium iodide (PI), and analyzing cellular DNA content by flow cytometry. Immunofluorescence techniques include detection of specific cell cycle indicators such as, for example, PCNA, 25 thymidine analogs and cyclins, with fluorescent antibodies.

**(i) Cytometric/Immunocytochemical methods**

Cell phase analysis by cytometry can be used to identify the distribution of cells in particular phases of the cell cycle, to determine the kinetics of progression through the phases and to monitor the molecular and functional mechanisms associated with the cell cycle, for 30 example by immunohistochemical detection of components such as cyclins, inhibitors of cyclin-dependent kinases, cell cycle-associated protooncogenes and tumor suppressor genes. In one method of cell phase analysis using flow cytometry, the nuclear DNA content of a cell can be quantitatively measured at high speed as an indicator of cell cycle phase. DNA

content is a marker of cell phase because the DNA content of a cell changes between the several phases of the cell cycle. Cells in G0/1 phase have DNA content set equal to 1 unit of DNA; cells in S phase duplicate DNA, increasing its content in proportion to progression through S; and upon entering G2 and then M phases, cells have twice the G0/1 phase DNA content (i.e., 2 units of DNA). Thus, S phase cells have a DNA content that is intermediate between that of cells in G1 and G2/M (which have twice as much DNA as cells in G1).  
5 Univariate analysis of cellular DNA content allows discrimination of G0/1, S and G2/M phase cells.

Flow cytometry measurement of cellular DNA content typically involves addition of a  
10 dye that binds stoichiometrically to DNA to a suspension of permeabilized cells or nuclei. Generally, cells are fixed or permeabilized, e.g., with a detergent, and then stained with a DNA-binding dye. Examples of such dyes include, but are not limited to, a nucleic acid-specific fluorochrome, propidium iodide (PI) or 4 '6 '-diamidino-2-phenylindole (DAPI). PI stains RNA in addition to DNA; thus, to avoid inclusion of measurement of fluorescence due  
15 to RNA in determining DNA content of a cell, it may be necessary to remove RNA by incubation with RNase. The DNA-bound PI emits red fluorescence when excited with blue light (488 nm). The DAPI-DNA complex can be excited by UV light (360 nm) and emits blue fluorescence. DNA can also be stained in live cells with the UV light-exitable fluorochrome Hoeschst 33242 which also emits blue fluorescence. Other DNA-binding dyes  
20 include, but are not limited to, Hoechst 33258, 7-AAD, LDS 751, and SYTO 16 (see, e.g., Molecular Probes Handbook of Fluorescent Probes and Research Chemicals, Haugland, Sixth Ed.; chapters 8 and 16 in particular).

In general, the DNA-binding dye is contacted with a cell (e.g., in concentrations ranging from about 1 µg/ml to about 5 µg/ml) and taken up passively by the cell. The dye is  
25 allowed to incubate for some period of time which can depend in part on the particular dye being used and can be determined empirically. Once inside the cell, the dye binds to DNA, for example by intercalation, although in some cases the dye can be a major or minor groove binding compound).

The stained material incorporates an amount of dye proportional to the amount of  
30 DNA. The stained material is then measured in the flow cytometer and the emitted fluorescent signal yields an electronic pulse with a height (amplitude) proportional to the total fluorescence emission from the cell. The results of fluorescence measurements can also be displayed as cellular DNA content frequency histograms which show the proportions of cells

in the various phases of the cycle based on differences in fluorescence intensity. Software containing mathematical models that fit the DNA histogram of a singlet have been developed to calculate the percentages of cells occupying the different phases of the cell cycle. Several manufacturers provide software for cell cycle analysis, including, for example, Becton Dickinson (CellFit<sup>TM</sup>).

Frequency histograms do not provide information about the rate of cell cycle progression or cell kinetics. However, the duration of a phase of the cell cycle ( $S; T_S$ ) can be estimated if the duration of the cell cycle ( $T_C$ ) or the doubling time of cells in culture is known. For example, the following equation may be used to estimate the duration of the S phase:  $T_S/T_C = \ln(F_S + 1)/\ln 2$ , where  $T_S$  is the duration of the S phase,  $T_C$  is duration of the cell cycle (or cell doubling time in culture) and  $F_S$  is a fraction of the S phase cells estimated from the DNA content frequency histogram. This equation applies to cells growing exponentially, when both daughter cells subsequently divide.

Univariate DNA content analysis also cannot be used to distinguish noncycling, quiescent G0 cells (e.g., normal quiescent cells that have not been stimulated by a mitogenic signal to enter the cell cycle and stem cells that can remain in G0 for long periods of time) because G0 cells have the same DNA content as cycling G1 cells. One way to distinguish cells in G0 and G1 phases is based on differing RNA contents of cells in these phases. G0 cells have a low RNA content reflecting a low number of ribosomes in the cells. Furthermore, cells in early and late stages of the G1 phase contain differing amounts of RNA. Cells in early G1 or G1A, i.e., G1 cells in the growth phase, accumulate RNA and proteins but have an amount of RNA that is less than a threshold amount required in order for a cell to enter S phase. Thus, cells in the early G1 phase are referred to as a sub-threshold subpopulation. Cells in late G1 or G1B, which can enter the S phase without additional RNA or protein accumulation, have supra-threshold amounts of RNA.

Bivariate cytometric analysis of DNA versus RNA content of cells allows discrimination between G1 and G0 cells, as well as between G1A and G1B cells, based on differences in RNA content. One method for differentially staining DNA and RNA exploits the metachromatic property of the cationic fluorochrome acridine orange (AO), which is excitable by blue light. Acridine orange interacts with DNA under specific staining conditions (proper concentration, ionic strength and pH) such that the DNA-AO intercalative complexes in the cell fluoresce green (530 nm). Under the same conditions, the RNA-AO complexes emit red (>640 nm) fluorescence. Acridine orange staining thus differentially

stains DNA and RNA (green vs. red, respectively), and can be used in conjunction with analysis by flow cytometers having a single-laser (488 nm) excitation. If a two-laser excitation cytometer is used in the analysis of cells for varying RNA content, DNA and RNA can be distinguished by differential staining with a combination of Hoechst 33242 and 5 pyronin Y [see, e.g., Shapiro (1998) *Cytometry* 2:143-150].

Another way to distinguish noncycling and cycling cells is based on differential expression of numerous proteins, such as, for example, proliferation-associated proteins. Antibodies against these marker proteins can be used for immunocytochemical identification of noncycling and cycling cells. For example, the antibody Ki-67 detects a protein that is 10 ubiquitous in cycling but frequently absent in noncycling cells [see, e.g., Endl *et al.* (2001) *Meth. Cell Biol.* 63:399-418]. The expression of protein reactive with Ki-67 varies during the cell cycle such that it is minimal in G1 cells and increases rapidly during S and G2 phases to reach a maximal amount in M cells. Proliferating cell nuclear antigen (PCNA), which is a co-factor of DNA polymerase  $\Delta$ , is another proliferation-associated marker protein in cells. 15 PCNA is specifically expressed in S-phase nuclei and is in the cytosol during G1 and G2/M [see, e.g., Larsen *et al.* (2001) *Meth. Cell Biol.* 63:419-432]. Cytometric analysis of an immunocytochemically detectable marker protein can be used in conjunction with DNA content analysis to reveal the cell cycle phase specificity of marker expression.

Univariate DNA content analysis also cannot be used to distinguish cells in G2 and M 20 phases of the cell cycle, which have the same DNA content. There are, however, numerous markers for mitotic cells that have been adapted to cytometric methods that can be used to distinguish such cells from G2 phase cells. For example, histone H3 phosphorylation, which varies throughout the cell cycle, is a useful marker for mitotic cells. From prophase until telophase nearly all histone H3 molecules in chromatin are phosphorylated on serine-10, 25 while during the remainder of the cell cycle only a small fraction of nucleosomes have phosphorylated H3. Antibodies that specifically recognize the phosphorylated epitope of histone H3 are available and can be used in immunocytochemical detection of phosphorylated H3 in conjunction with flow cytometric methods [see, e.g., Juan *et al.* (2001) *Meth. Cell Biol.* 63:343-354]. Bivariate analysis of DNA content versus phosphorylated 30 histone H3 provides for identification of all four phases of the cell cycle.

In another method of cell phase analysis using flow cytometry, cellular levels of cyclin proteins can serve as the basis for determining the phase of a cell and for distinguishing cells in different phases. Progression through the cell cycle is maintained by

timely, sequential phosphorylation and dephosphorylation of several intracellular proteins. Cyclins are proteins involved in the cell cycle control system in cells which works by phosphorylation of proteins to active or inactive forms. Cyclins bind to and activate cyclin-dependent kinases (cdks). Cyclin-dependent protein kinases influence the progression of the cell cycle by phosphorylating serine and threonine residues on select proteins that control the synthesis of cellular elements required for reproduction. Mammalian cells appear to contain separate cdk5 proteins that are specific for the G1 and G2 checkpoints through which a cell passes prior to progressing into the S and M phases, respectively.

Distinct classes of cyclins are synthesized during the G1 and G2 phases. In the normal vertebrate cell cycle control system, at least eight cyclins (cyclins A-H) and eight cdk5 proteins (cdc2 and CDK 2-8) are known. G1 cyclins (e.g., cyclins D and E) accumulate and bind cdk5 proteins during the G1 phase, and a different class of cyclins, mitotic cyclins, accumulate and bind cdk5 proteins during the G2 phase. For example, cell cycle progression can begin with induction of cyclin D; passage of the G1 checkpoint requires the binding of CDK2 protein with cyclin E; activation of DNA synthesis requires CDK2 protein binding with cyclin A, and entry into M phase requires formation of a complex between cdc2 and cyclin B [see, e.g., Merrill (1998) *Meth. Cell Biol.* 57:229-249].

The cellular level of cdk5 proteins is substantially invariable during the cell cycle. In contrast, cyclins are synthesized and degraded as the cell progresses through the cell cycle. Following synthesis, cyclins bind to cdk5 proteins promoting the phosphorylation of target proteins by the cdk5. Subsequently, the cyclins are degraded, the cdk5 are released and target protein phosphorylation is reduced or ceased. The rate-limiting step in the binding reactions between cdk5 and cyclins appears to be the synthesis of the appropriate cyclin. Thus, the cellular content of some of the cyclins, for example, cyclins D, E, A and B, oscillates detectably during the cell cycle. Cyclin expression can be analyzed immunocytochemically (e.g., using fluorescently labeled anti-cyclin antibodies) by flow cytometry and used in distinguishing cells in different phases of the cell cycle based on bivariate analysis of cyclin expression versus DNA content. Correlation of DNA content with changes in cyclins permits visualization of nine subdivisions of the cell cycle [see, e.g., Darzynkiewicz *et al.* (1996) *Cytometry* 25:1-13].

Bivariate analysis of DNA content versus 5-bromo-2'-deoxyuridine (BrdU) incorporation is another cytometric assay for assessing cell phase [see, e.g., Dolbeare *et al.* (1983) *Proc. Natl. Acad. Sci. U.S.A.* 80:5573-5577; Moran *et al.* (1985) *J. Histochem.*

*Cytochem.* 33:821-827; Beisker *et al.* (1987) *Cytometry* 8:235-239; Gray *et al.* (1990) "Quantitative cell cycle analysis," in *Flow Cytometry and Sorting*, Melamed, Lindmo and Mendelsohn, eds., pp. 445-467; and *Current Protocols in Cytometry* (1997), Unit 7.7, John Wiley & Sons, Inc.]. BrdU is a thymidine analog that is incorporated into DNA during replication in cells exposed to the analog. DNA that has incorporated the analog can be detected immunocytochemically using fluorescein-tagged anti-BrdU antibodies. DNA content may be assessed, for example, by counterstaining with a red fluorescing intercalating fluorochrome such as, for example, PI or 7-aminoactinomycin D (7-AAD). Bivariate analysis of DNA content versus immunofluorescence of anti-BrdU antibody distinguishes S phase cells on the basis of their difference in DNA content from G1 or G2/M cells and also based on incorporation of the green fluorescing anti-BrdU antibodies.

(ii) **Centrifugation/Elutriation-based methods**

Centrifugation and centrifugal elutriation can be used to fractionate cells according to their size. Because cells in different phases differ in size, these methods can also be used to sort cells by cell phase and to thereby assess the phase of a cell. For example, early G1 phase cells are about half the size of mitotic or late G2 cells.

(iii) **Chromosome phase**

Chromosomes undergo morphological, ultrastructural and topological changes during progression of the cell cycle. Thus, chromosomes from cells in different phases of the cell cycle can be distinctive. The topology of a chromosome differs at different phases of the cell cycle. Interphase chromosomal DNA exists in various decondensed states to facilitate gene expression. Chromatin in chromosomal regions that is not being transcribed exist predominantly in the condensed form while regions being transcribed assume an extended form. Within S phase, chromosomal DNA is further dispersed as it unwinds during the replication process. Upon conclusion of S phase cohesion occurs to keep extended sister chromatids tightly associated. Typically, chromosomes begin to condense during prophase, undergoing several orders of supercoiling guided by histones and other facilitator proteins. Chromosomes are most dense during metaphase and begin to decondense again during telophase as the sister cells divide and normal transcription levels resume. Due to the various states in which chromosomal DNA exists throughout the cell cycle, when delivering DNA, particularly chromosomes, functional fragments thereof or artificial chromosomes into cells as provided herein, the phase of the isolated chromosome as well as the recipient cell

chromosome can be a consideration in providing for improved transfer into the cells.

Chromosomal phase can be determined by the methods described herein.

**(b) Arresting cell cycle**

**(i) Agents and methods of arresting cell cycle**

5 Cell arrest agents may be used to increase the amount of cells in a particular cell cycle phase within a population of cells or to arrest a particular cell at a particular phase. The agent may then be removed to allow the cell(s) to proceed through the cell cycle. Upon release from the agent, a population of cells will proceed more or less simultaneously through about one cell cycle. Arrest agents for use in the methods herein include compositions, conditions  
10 and physical treatments that arrest the cell cycle in a particular phase. Such agents include, but are not limited to, compounds and chemical compositions, peptides, proteins, temperature, light, pH, radiation and pressure.

Physical conditions, for example, may include but are not limited to starvation techniques such as nutrient, growth factor or hormone deprivation. Such techniques lead to  
15 an accumulation of cells in a particular stage, usually the G1 or G0 stage. Re-addition of nutrients or growth factors then allows the cells to re-enter the cell cycle in synchrony for about one generation. Most proliferating cell lines are known to respond to nutrient, growth factor or hormone deprivation in this manner. Two examples include the L929 mouse fibroblast and Nb2 rat lymphoma cell line. Culturing the L929 mouse fibroblast under serum  
20 starvation conditions (Glasgow Modified Minimal Essential Medium with 0.5% newborn calf serum and without glutamine) results in arrest of cells G0 phase. Upon replacing the starvation medium with normal growing medium, all cells progressed through the cell cycle in a synchronized manner (see Marenzi *et al.* (1999) *Molec. Biol. Reports* 26:261-267). The Nb2 rat lymphoma cell line is absolutely dependent on prolactin for stimulation of  
25 proliferation (see Gout *et al.* (1980) *Cancer Res.* 40:2433-2436). Culturing the cells in prolactin-deficient medium for 18-24 hours leads to arrest of proliferation, with cells accumulating early in the G1 phase of the cell cycle. Upon addition of prolactin, all the cells progress through the cell cycle until M phase at which point greater than 90% of the cells would be in mitosis. Deprivation techniques may be combined with additional arrest agents  
30 including other physical conditions, compounds or compositions, to increase the percentage of synchronized cells in the population. One or more agents may be used simultaneously or in succession to affect cell arrest. Temperature is another physical cell cycle arrest agent. For example, maintaining V79 cells at 25-32°C results in an accumulation of cells in G0/G1.

A further physical arrest agent is incubation under pressurized nitrous oxide which results in arrest at M phase (Brinkley and Rao (1973) *J. Cell Biol.* 58:96-106)

Cell arrest compounds for use in the methods provided herein are available commercially or can be synthesized by those of skill in the art. Cell arrest compounds typically target specific phases of the cell cycle. S phase arrest agents include, among others, agents that affect DNA replication or earlier phases through action on DNA polymerase and ribonucleotide reductase. Aphidicolin, 5-aminouracil (a thymidine analogue) thymidine, and hydroxyurea are examples of S phase arrest agents. Hydroxylurea disrupts S phase, for example, by destroying an essential tyrosine-free radical on the ribonucleotide reductase enzyme. Thymidine causes overaccumulation of thymidine triphosphate, which allosterically inhibits the reduction of cytidine diphosphate by ribonucleotide reductase, thus starving the replication forks for deoxycytidine triphosphate. Aphidicolin blocks replication by directly bidding and inhibiting DNA polymerase. M phase cell arrest can be mediated by agents that affect specific mitotic events such as microtubule polymerization and spindle arrest. M phase arrest agents include, for example, nocodazole, colchicine, demecolcine (colcemide), oryzalin, propyzamide, trifluralin, 8-hydroxyquinoline, paradichlorobenzene, vinblastine, and a number of other glucosides, alkaloids, and coumarins (see Sharma, AK (1956) *Bot Rev* 22:665-695; Sharma AK and Sharma A (1999) *Plant Chromosomes: Analysis, Manipulation, and Engineering*; Sharma AK (1999) *Methods Cell Sci* 21:73-78). Other arrest compounds for use in the methods provided herein include but are not limited to 1- $\beta$ -D-Arabinofuranosylcytosine, olomoucine, roscovitine, 2-[N-(2-hydroxyethyl)]-N-(4-methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine, daidzein, camptothecin, 5,6-Dichloro-1- $\beta$ -D-ribofuanosylbenzimidazole, 5-fluorouracil, dexamethasone, acycloguanosine, calpain inhibitor I, cytosine arabinoside, CeReS-18, trichostatin A, indole-3-methanol, Staurosporine, tryprostatin B, and verruculogen.

Any cell arrest agent or combination of arrest agents can be used for enhancing a particular method of delivering nucleic acid molecules, such as DNA, into a particular cell type using the provided methods. The amount of agent and duration of exposure of a cell to the agent is dependent on the agent and cell type. These parameters can be readily determined for any agent and any cell by the methods provided herein.

In a particular embodiment of the method, one arrest agent is applied in an optimal amount and for an optimal duration to affect cell arrest. In another embodiment, one arrest agent is applied and removed in successive rounds or applied in different concentrations at

different time points throughout the cell cycle to effect cell arrest. For example, using a "double block" protocol, cells may be incubated in replication-inhibiting levels of an arrest agent, such as thymidine, for sufficient time to let G<sub>2</sub>, M, and G<sub>1</sub> cells collect in early S, then cells are incubated in low levels of agent for sufficient time to let all S phase cells complete 5 replication, and finally cells are incubated again in high levels of agent for sufficient time to allow all the newly generated G<sub>2</sub>, M, and G<sub>1</sub> cells to collect in early S phase. In another embodiment two or more arrest agents are applied simultaneously to effect cell arrest. In a further embodiment, two or more agents are applied successively to effect cell arrest. For example, to boost the yield of cells arrested in M phase, cells can be first exposed to an S 10 phase arrest agent such as aphidicolin or thymidine. The S phase block can then be removed and the cells further incubated under mitosis-arresting conditions. After arresting with the S phase agent, a greater number of the cells in the population will be in a relatively late cell cycle phase, thus a greater number of cells will collect in M phase during the latter incubation.

15 One of skill in the art by using the provided optimization methods can readily determine which of the cell arrest agents or combination of agents are best suited for facilitating cell arrest and enhancing delivery of specific nucleic acid molecules, such as DNA, into a specific target cell type. These methods include determining the mean generation time, lengths of each phase of the cell cycle, fraction of cells proliferating in a cell 20 population, arrest agent concentration, arrest agent incubation time, growth conditions, and nutrient conditions

Cell arrest protocols may include contacting cells with arrest agents for periods of time related to the duration of specific cell cycle phases, for example, the double block method described above. Thus, optimization may require knowledge of the duration of each 25 cell cycle phase. To determine the duration of each cell cycle phase, determination of the mean generation time (length of one cell cycle) may be required. The mean generation time of cells in a population can be calculated from the change in cell number with incubation time. For example, cell number as a function of incubation time may be plotted for several different time points on semilog paper. A best fit line can be drawn through the data points 30 and the time take for cell number to double can be calculated, yielding the mean generation time.

If the mean generation time is known, the duration of G<sub>1</sub>, S, G<sub>2</sub>, and M phase may be estimated. The duration of each cell cycle phase may be estimated, for example, by

microscopy or flow cytometry. Microscopic methods may include autoradiography of labeled mitotic figures after pulse/chase labeling with [<sup>3</sup>H]thymidine (see e.g., Quastler and Sherman (1959) *Exp. Cell Res.* 17:420-438). When using flow cytometry, asynchronously growing cells can be stained with a DNA-intercalating dye such as propidium iodide, and 5 fluorescence intensity can be used to determine the frequency of cells with a 2n, intermediate, or 4N complement of DNA. From a histogram of the DNA values, the phases can be determined manually or by computer algorithm (see e.g., Merrill, G. (1998) *Methods Cell Biology; Cell Synchronization* 57:229-249).

In addition, knowing the fraction of cells that are proliferating in the cell population 10 may be useful for optimizing cell arrest conditions. Many methods of analyzing the average duration of a cell division cycle and its component phases assume that most cells (>95%) in the population are actively proliferating and that cell death is low (<5%). If a significant fraction of a population is quiescent, then the amount of quiescent cells may need to be considered when determining cell cycle kinetics of a cell population. To determine the 15 fraction of quiescent cells in a population, the number of cells that fail to replicate their DNA during an interval corresponding to twice the mean generation time can be calculated. To determine the replicative fraction, [<sup>3</sup>H]thymidine incorporation followed by autoradiography or bromodeoxy-uridine(BUdr)incorporation followed by immunostaining may be used.

The optimum arrest agent concentration is the concentration that results in the greatest 20 percentage of synchronized cells while maintaining acceptable levels of viability. Optimum arrest agent concentration may be determined by monitoring the dose response of a specific cell to a specific arrest agent. Dose response can be determined by titrating cells with an arrest agent and detecting cellular response. Cellular response can be detected, for example, by monitoring cell cycle phase and cell viability for each dose point over a period of one 25 generation. Cell cycle phases can be monitored using cytometric analysis. In a specific example, for each dose point, cells are exposed to an agent or stain such as propidium iodide and the proportion of cells in G<sub>0</sub>-G<sub>1</sub>, S, G<sub>2</sub>, and M are determined by flow cytometry. PI binds to the DNA within a cell and the amount of DNA contained in a cell is determined by comparing experimental PI measurements with control PI measurements. Any dye that 30 incorporates into DNA can be used in a similar manner to PI. A cell that has not undergone DNA synthesis (S phase of the cell cycle) has 1 copy of the genome and is therefore in G<sub>0</sub>/G<sub>1</sub>. Once S phase is initiated the amount of DNA is greater than one copy and at the end of S phase the amount of DNA is at 2 copies and the cell is then in the G<sub>2</sub>/M stage of the cell

cycle. Following nuclear and cellular division (M phase), each cell again has a single copy of the genome.

Arrest agent incubation time is a key factor for optimizing cell arrest. Excessively long incubations lead to poor synchrony or low viability. Insufficiently long incubations lead to poor synchrony because not all cells in the population reach the arrest state. Optimum arrest agent incubation time can be determined by exposing the cell to the agent and monitoring cell cycle phase over a period of one generation as described above. The optimum arrest agent incubation time is the time point at which the majority of the cells are arrested in the same phase while maintaining acceptable levels of viability.

10 Growth and nutrient conditions are dependent on cell type. Methods of determining growth and nutrient conditions are well known to those of skill in the art.

Meiosis regulating agents may also be used to obtain cells in a preselected phase. Meiosis regulating compounds are for example disclosed in U.S. Patent No. 6,486,145; 6,407,086; and 5,716,777 (all of which are incorporated herein by reference).

15 (ii) **Assessing cell cycle arrest**

Extent of cell cycle arrest can be determined using any methods of assessing cell phase as described herein or known in the art.

(c) **Releasing cells from cell cycle arrest**

In the methods provided herein for introducing nucleic acid molecules into cells, the 20 nucleic acid molecules can be introduced into cells whose growth has been arrested, or into cells at a defined time point after release from cell cycle arrest. Release of cells from cell cycle arrest results in a synchronous population of cells, thus the defined time point corresponds to a particular phase of the cell cycle in the synchronous population of cells. Methods for releasing cells from cell cycle arrest involve the restoration of normal growth 25 conditions. Such methods are dependent on the cell arrest agent and type of cell used. For example, cells arrested by physical conditions such as nutrient, growth factor or hormone deprivation, are released from arrest by re-addition of the nutrient, growth factor, or hormone to the growth media. Cells arrested with a chemical agent may be released by removing the agent by washing and/or replacing the media as described, for example, in Example 10, or by 30 the addition of an agent that counteracts or inhibits the arrest agent, for example, adding 2'-deoxycytidine 5'mono-phosphate (dCMP) to release cells from thymidine arrest. Upon restoration of normal growth conditions the cells re-enter the cell cycle in synchrony for about one generation depending on the cell type. The cells then gradually return to their

logarithmically growing state. Re-entry into the cell cycle may be monitored by the methods described herein for monitoring cell cycle phase.

(d) Assessing delivery efficiency

Rapid Assessment Methods

- 5       Rapid delivery assessment methods based on automated, sensitive and accurate analysis procedures, such as flow cytometry, can be used to evaluate delivery efficiency. Such methods are less time-consuming and more accurate than manual detection of individual transfected cells by microscopic techniques. Using such rapid methods, it is possible to analyze nucleic acid molecule delivery data within 48 hours after transfection.
- 10      Microscopic and colony formation analysis methods that may be used in evaluating stable nucleic acid molecule delivery rely on manual visualization or measurement of nucleic acid molecules (e.g., a selectable marker gene) expression, which is a distinct process from delivery. Such methods are associated with time delays in obtaining an assessment of the delivery method.
- 15      Data collected by flow cytometry analysis are statistically superior due to the ease at which large numbers of events, e.g., nucleic acid molecule transfer, are collected. The positive values obtained in these methods are instrument derived and therefore not as susceptible to judgment errors. Thus, these methods provide for greater accuracy in assessing nucleic acid molecule delivery. In contrast, microscopic analysis is limited by the time involved for scoring positive events and sample size is restrictive.
- 20      Because the methods of monitoring nucleic acid molecule delivery detect labeled nucleic acid molecules, such as DNA, and not a reporter gene expression product, it is possible to measure absolute values of nucleic acid molecules transferred, within twenty-four hours, without being hindered by cell autofluorescence and by the problems of differentiating wild-type cells from cells expressing low levels of reporter gene products (see, e.g., Ropp *et al.* (1995) *Cytometry* 21:309-317).
- 25      In rapid methods for monitoring nucleic acid molecule delivery, the nucleic acid molecules, such as DNA, to be delivered are labeled to allow for detection of the nucleic acid molecules in recipient cells after transfer into the cells. The nucleic acid molecules may be labeled by incorporation of nucleotide analogs. Any nucleic acid molecule analog that may be detected in a cell may be used in these methods. The analog is either directly detectable, such as by radioactivity, or may be detected upon binding of a detectable molecule to the analog that specifically recognizes the analog and distinguishes it from nucleotides that make

up the endogenous nucleic acid molecules, such as DNA, within a recipient cell. Analogs that are directly detectable have intrinsic properties that allow them to be detected using standard analytical methods. Analogs may also be detectable upon binding to a detectable molecule, such as a labeled antibody that binds specifically to the analogs. The label on the 5 antibody is one that may be detected using standard analytical methods. For example, the antibody may be fluorescent and be detectable by flow cytometry or microscopy.

In particular embodiments of these methods, the nucleic acid molecules, such as DNA, to be transferred is labeled with thymidine analogs, such as iododeoxyuridine (IdUrd) or Bromodeoxyuridine (BrdU). In preferred embodiments, IdUrd is used to label the nucleic 10 acid molecules, such as DNA, to be transferred. The transferred IdUrd-labeled nucleic acid molecules, such as DNA, may be immunologically tagged using an FITC-conjugated anti-BrdU/IdUrd antibody and quantified by flow cytometry. Thus, the transfer of the labeled nucleic acid molecules, such as DNA, into recipient cells can be detected within hours after transfection.

15 **Microscopy-based Assessment Methods**

Microscopic techniques for visualizing chromosome or plasmid transfer using bromodeoxyuridine (BrdU) (see, e.g., Pittman *et al. J Immunol Methods* 103:87-92 (1987)) are known in the art. These methods typically involve use of a large sample size to detect any low levels of transfer and further involve manual scoring.

20 **Factors to Consider in Addressing Delivery of Nucleic Acids**

Delivery of nucleic acids, including DNA, into cells is a process in which nucleic acids are transferred to the interior of a cell. Methods for the delivery of nucleic acids may be assessed in a variety of ways, including the following.

(i) **Transfer or delivery efficiency**

25 A delivery method may be assessed by determining the percentage of recipient cells in which the nucleic acids, including DNA, is present (i.e., the transfer efficiency). However, when evaluating a delivery method for the ultimate goal of generating cells that express the transferred nucleic acid, there are additional factors beyond mere presence of the nucleic acid in recipient cells that should be considered. Included among these additional factors is 30 integrity of the nucleic acids and cell viability. When assessing a proliferating cell population, clonogenicity is the method of choice to measure viability. When the target cells population is non-dividing or slow growing, metabolic integrity can be monitored.

### (ii) Clonogenicity

Clonogenicity represents a measure of the survivability of cells with respect to a delivery procedure, growth conditions and cell manipulations (e.g., plating). It is important to assess clonogenicity to determine whether a delivery procedure results in a sufficient 5 number of viable cells to achieve a desired number of cells containing the transferred nucleic acid.

Clonogenicity may be expressed as a clonal fraction. The clonal fraction is an index that is calculated by multiplying two separate fractions and normalizing to a control plating efficiency correction factor (CPE). The two separate fractions that are multiplied in this 10 calculation are the fraction of cells that survive a delivery procedure (population cell yield) and the fraction of cells that survive a plating procedure. The calculation is thus as follows:

$$\text{Colonial Fraction} = \frac{\# \text{ viable colonies after plating}}{\# \text{ cells plated}} \times \frac{\# \text{ cells post-transfection}}{\# \text{ cells transfected} \times \text{CPE}}$$

15

The values used in this calculation for the number of cells post- transfection (i.e., post-delivery) and the number of colonies post-plating is based on cell or colony numbers at certain times in the process. For instance, the value for the number of cells post-transfection is representative of the number of cells at a time after nucleic acid delivery that is sufficient 20 for the delivery process to be completed. This time may be determined empirically. Typically this time ranges from 4-48 hours and generally is about one day after transfection. Likewise, the value of the number of viable colonies post-plating is representative of the number of colonies at a time after nucleic acid delivery that is sufficient for the non-viable 25 cells to be eliminated and the viable cells to be established as colonies. This time may be determined empirically. Typically this time ranges from that in which the average colony is made up of approximately 50 cells or generally is a time at which five cell cycles have passed.

A correction factor is included to take into account the plating efficiency of control wells, which is the ratio determined by the number of colonies counted divided by the 30 number cells initially plated (typically 600-1000 cells). For LM(tk-) and V79-4 cells, the value of the correction factor typically ranges from about 0.7 to about 1.2 and may be, for example, 0.9.

The number of cells plated should remain constant at 1000 (simplified plating efficiency assay) done in duplicate, except in the case where the CPE is below 0.3, then

number of cells seeded should be increased to a range of 5,000-50,000. If the CPE is below 0.1-0.2, then a viable fraction analysis should be considered.

**(iii) Viable fraction**

If the target cell population is non-dividing or slowly dividing then reproductive or 5 clonogenicity assays are not relevant. Less direct measurements of cell viability must be used to measure cell killing that monitor metabolic death rather than loss of reproductive capacity. These procedures include, for example: (1) membrane integrity as measured by dye exclusion, (2) inhibition of nucleic acid synthesis as measured by incorporation of nucleic acid precursors, (3) radioactive chromium release, and (4) MTT ASSAY (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide). These methods are different from 10 measurements of loss of proliferative capacity, as they reflect only immediate changes in metabolism, which can be reversed or delayed and hence lead to errors in estimation of cell viability. To minimize these errors, correlation of duplicate procedures is suggested.

**(iv) Potential transfection efficiency (PTE) and determination of 15 Chromos Index (CI)**

In assessing a delivery method used to transfer nucleic acids to cells with the goal of expression of the nucleic acids, including DNA, therein, it is desirable to obtain an indication 20 of the theoretical maximum percentage of cells that are viable and contain the nucleic acid out of the total number of cells into which nucleic acids were delivered. This is referred to as the potential transfection efficiency and may be calculated from existing or historical experimental data sets and is determined as follows:

$$\text{Potential Transfection Efficiency (PTE)} = \text{Transfer Efficiency} \times (\text{Clonal Fraction or Viable Fraction}) \times \text{correction factor (CF)}$$

25

The Chromos Index (C.I.) is an effective and rapid method to determine the Potential Transfection Efficiency of a proliferating population by using experimental values of % labeled nucleic acid, such as ACes, delivery to measure transfer efficiency and clonal fraction measured using a simplified clonogenicity assay.

30

$$\text{Chromos Index (CI)} = \% \text{ labeled ACes delivery} \times \text{estimated Clonal fraction} \times \text{CF}$$

The values of the transfer efficiency and of the clonal fraction and viable fraction are calculated as described above. The correction factor (CF) takes into account sample size,

sample time and control plating efficiency. If all these factors are constant for each variable i.e., sampling time and size then the correction factor will approach the inverse of the value for the C.P.E., i.e., such that the clonal fraction or transfer efficiency can still approach 100% even with a low CF, or in other words, if delivery and viability are 100%, then the maximum  
5 potential transfection efficiency will equal the plating efficiency of the control cells. The calculation of C.I. allows for determination of each variable optimization, with the goal being for parameters, such as transfer efficiency, clonal fraction, and CF to approach one (or 100%). If sample size or time varies for either clonal fraction or transfer efficiency, then CF represents the extrapolated value based on slope or rate of change. An application of this  
10 assessment is provided in the EXAMPLES.

A stable transfection efficiency of about 1% is in the range (1-100%) that is considered useful for the introduction of large nucleic acid molecules into target cells. It is possible, using methods provided herein, to predict which delivery methods have to be selected for achieving desired transfection efficiencies without having to grow transfectants  
15 for extended times under selective conditions and determine numbers of cells surviving selection marker expression. This analysis involves calculation of the Chromos Index (CD) which integrates a "biological" value (the clonal fraction) with a measurement of chromosomal "uptake" or transfer efficiency (percentage of cells containing delivered ACes).

**(e) Assessing integrity of transferred nucleic acid**

Assessment of delivery of nucleic acid molecules may also be combined with an assessment of nucleic acid molecule, such as DNA, expression and integrity in recipient cells to provide even further information concerning the overall process of nucleic acid molecule transfer for purposes of expression.  
20

It is of interest to evaluate the stability of the nucleic acid molecule, such as DNA,  
25 under the selected delivery conditions. Some delivery conditions and agents may have adverse effects on nucleic acid molecule structure. Furthermore, the labeling techniques used in certain methods of monitoring nucleic acid molecules, such as DNA, delivery may also impact nucleic acid molecules, such as DNA, structure and function.

The effects of delivery conditions on nucleic acid molecules may be assessed in a  
30 variety of ways, including microscopic analysis. In a particular exemplary analysis of the stability of artificial chromosomes, e.g., ACes, the chromosomes are exposed to the conditions of interest, e.g., IdU labeling, and analyzed under a fluorescent microscope for the ability to remain intact and condensed after incorporation of nucleotide analogs.

Analysis of the transfected cells may be performed to identify and assess the integrity of transferred nucleic acids. For example, the cells may be analyzed for indications of transferred nucleic acids such as artificial chromosomes or chromosomal segments, the presence of structures that may arise in connection with such nucleic acids and/or the 5 presence of such nucleic acids. The size and structure of a transferred nucleic acid such as an artificial chromosome or ACes may also be determined. Analysis of the cells typically involves methods of visualizing nucleic acid structure and particularly chromosome structure, including, but not limited to, G- and C-banding and fluorescent in-situ hybridization (FISH) analyses using techniques described herein and/or known to those of skill in the art. Such 10 analyses can employ specific labelling of particular nucleic acids, such as satellite DNA sequences, heterochromatin, rDNA sequences and heterologous nucleic acid sequences.

**(f) Assessing expression of transferred nucleic acid**

To facilitate analysis of nucleic acid molecules, such as DNA, expression, it is desirable to include in the transferred nucleic acid molecules, such as DNA, a reporter gene 15 that encodes a readily detected product. For direct detection, such reporter gene products include, but are not limited to green fluorescent proteins (GFP), Red Fluorescent protein (RFP), luciferases, and CAT. For indirect detection, reporter gene products include, but are not limited, to  $\beta$ -galactosidase and cell surface markers.

By using, for example, artificial chromosomes such as ACes containing a GFP 20 reporter gene, such as, but are not limited to, GFP coding sequences in combination with labeling of the ACes with DNA analogs, such as IdU, delivery and expression can be rapidly and accurately monitored. For example, following the delivery of IdU-labeled GFP gene-containing ACes to target cells by any of the described methods, the cells containing the 25 ACes are split into two populations. One population is fixed and stained for IdU and analyzed by flow cytometry to determine percentage delivery. The other population is allowed to go through 4-5 cell divisions (approximately 72 hours), and the GFP fluorescence is measured as an indication of expression.

Such studies have revealed that incorporation of the analog label does not affect GFP 30 protein expression, which indicates that the methods may be combined to monitor delivery and early expression of the ACes, thus providing more information to rapidly evaluate the efficiency of delivery methods. The combined methods can also be used to map the biological events between the initial stages of delivery and early gene expression.

(g) Cell cycle synchronization

The methods described herein provide for the transfection of a single cell or a population of two or more cells. To transfect a population of cells, it is often desirable to use a synchronous population of cells. A synchronous population of cells refers to a population 5 in which a greater number of cells are in the same single phase compared to a corresponding asynchronous population. An asynchronous population of cells can be a population of cells in conditions that allow for normal exponential growth that have not been exposed to conditions that effect synchronization. Any method for obtaining a synchronous population of cells may be used in the methods described herein.

10 Methods for obtaining a synchronous population of cells may include isolating cells at a specific cell cycle stage using mechanical means such as mitotic detachment or elutriation or chemical means such as exposing the cell to a cell cycle arrest agent. Mitotic detachment (or mitotic shake-off) is a minimally disruptive way of synchronizing monolayer cells. When most monolayer cells enter mitosis, they round up and become only slightly attached to the 15 substratum of the culture plate. Because of their size and loose attachment, mitotic cells can be isolated by shaking and transferring the medium containing the detached cells to a fresh culture vessel. To generate large numbers of cells, a greater number of mother culture plates or vessels may be used.

Elutriation methods include sorting cells based upon morphological or biochemical 20 features such as cell size, chromosome morphology, DNA content, base content, protein content (including cyclins), or other indicators of cell cycle phase such as enzymes or other factors that are expressed, degraded, activated or inactivated at defined phases of the cell cycle. Cells may also be sorted using combinations of the above cell cycle phase indicators. Combinations of cell cycle phase indicators can be used simultaneously or in consecutive 25 sorting procedures.

Centrifugal elutriation is one method of sorting cells based on size and density. The system consists of a specialized centrifuge rotor in which the centrifugal force and opposing bulk medium flow create a gradient, with smaller cells at the top and larger cells at the bottom. The rotor speed or medium flow is manipulated such that the gradient of size-separated cells is pushed toward the top and the small cells at the top of the gradient are eventually pushed out of the elutriation chamber and into a collection vessel. With further manipulation of the rotor speed and medium flow, progressively larger cells are pushed out of the elutriation chamber. Since G1 cells are roughly half the size of mitotic or late G2 cells,

centrifugal elutriation can be used to fractionate cells according to their position in the cell cycle. Unlike the mitotic shake-off procedure, centrifugal elutriation can be used to synchronize both monolayer and suspension culture cells.

Cell phase sorting can also be accomplished through the use of a flow cytometer and

5 sorter such as the fluorescence-activated cell sorter (FACS) as described herein. After elutriation the number and volume of cells in different fractions can be assessed using a multichannel cell analyzer. An advantage of using physical means to create a synchronized population of cells is that the cells do not spend any time in a growth-arrested state and therefore, anabolic activity remains relatively constant. Such methods also avoid the possible  
10 side-effects from treatment of cells with chemicals.

Alternatively, cell synchronization agents may be used alone or in combination with physical sorting means to enhance a synchronous population of cells. Cell cycle arrest agents, as described herein, act by blocking the progression at a defined stage of the cell cycle which results in the accumulation of cells within the defined stage. Upon release from the  
15 arrest agent, the cells progress through the cell cycle in synchrony. When used in combination with cell sorting techniques, the cells may be sorted prior to or after release from the arrest agent.

The percentage of cell cycle synchronization within a population can be determined at different time points by the methods described above. The percentage of cell cycle  
20 synchronization is a measure of the percentage of cells cycling together in any one given phase at any one time point. The maximum percentage of cell cycle synchronization achievable is 100% of the cell population cycling together in any one phase. The percentage of cell cycle synchronization achievable using the cell cycle sorting and/or arrest and release methods described herein is at least 50%, at least 60%, at least 70% at least 75%, at least  
25 80%, at least 85%, at least 90%, at least 95% of cells cycling together in a particular phase.

#### D. METHODS FOR THE DELIVERY OF DNA INTO CELLS

In methods provided herein for delivering nucleic acids to cells, a recipient cell in a pre-selected phase is contacted with a nucleic acid molecule, such as a large nucleic acid molecule. The contacting can be conducted in a variety of ways under a variety of  
30 conditions. Conditions and procedures can be determined, for example, by comparing the delivery and/or transfection efficiencies (as can be calculated using methods described herein) achieved under various conditions.

The conditions used for delivering particular nucleic acid molecules, such as DNA, to recipient cells can depend on the particular nucleic acid molecule being transferred and the particular recipient cell. Particular conditions are those that result in the greatest amount of nucleic acid molecules, such as DNA, transferred into the cell nucleus with an acceptable degree of cell survival. Suitable conditions for delivery of particular pairings of nucleic acid molecules, such as DNA, and recipient cells can be determined using methods of monitoring nucleic acid molecules, such as DNA, delivery and methods of screening agents and conditions as provided herein or can be determined empirically using methods known to those of skill in the art.

In determining which conditions to use for contacting a nucleic acid with a cell in a pre-selected phase, a number of parameters can be considered. A method for detection of delivered nucleic acid is provided. This method, which can be used for assessing delivery of any nucleic acid molecule, can be used as a rapid screening tool to optimize nucleic acid, e.g., chromosome, transfer conditions.

In particular, delivery conditions can first be assessed for the ability to transfer nucleic acid molecules, such as DNA, into cells and to identify methods that provide a sufficient number of viable cells that express the transferred nucleic acid molecules, such as DNA. Once such conditions are identified, they can be optimized using delivery monitoring methods provided herein or known in the art and then assessed for the ability to provide for expression of the transferred nucleic acid molecules.

### **1. Naked DNA**

“Naked” nucleic acid can be contacted with the recipient cell in the absence of any delivery agent or other procedures designed to facilitate transfer of nucleic acids into cells.

### **2. Microinjection**

Nucleic acids can be delivered into cells through microinjection. Procedures for microinjection are known in the art.

### **3. Delivery agents**

Delivery agents include compositions, conditions and physical treatments that enhance contact of nucleic acid molecules, such as DNA, with cells and/or increase the permeability of cells to nucleic acid molecules, such as DNA. Such agents include, but are not limited to, cationic compounds, peptides, proteins, energy, for example ultrasound energy and electric fields, and cavitation compounds.

Delivery agents for use in the methods provided herein include compositions, conditions or physical treatments to which cells and/or nucleic acid molecules, such as DNA, can be exposed in the process of transferring nucleic acid molecules, such as DNA, to cells in order to facilitate nucleic acid molecules, such as DNA, delivery into cells. For example, compounds and chemical compositions, including, but not limited to, calcium phosphate, DMSO, glycerol, chloroquine, sodium butyrate, polybrene and DEAE-dextran, peptides, proteins, temperature, light, pH, radiation and pressure are all possible delivery agents.

### (a) Cationic compounds

Cationic compounds for use in the methods provided herein are available

10 commercially or can be synthesized by those of skill in the art. Any cationic compound can  
used for delivery of nucleic acid molecules, such as DNA, into a particular cell type using the  
provided methods. One of skill in the art by using the provided screening procedures can  
readily determine which of the cationic compounds are best suited for delivery of specific  
nucleic acid molecules, such as DNA, into a specific target cell type.

### (i) Cationic lipids

Cationic lipid reagents can be classified into two general categories based on the number of positive charges in the lipid headgroup; either a single positive charge or multiple positive charges, usually up to 5. Cationic lipids are often mixed with neutral lipids prior to use as delivery agents. Neutral lipids include, but are not limited to, lecithins; phosphatidylethanolamine; phosphatidylethanolamines, such as DOPE (dioleoylphosphatidylethanolamine), DPPE (dipalmitoylphosphatidyl-ethanolamine), POPE (palmitoyloleoylphosphatidylethanolamine) and distearoylphosphatidylethanolamine; phosphatidylcholine; phosphatidylcholines, such as DOPC (dioleoylphosphatidylcholine), DPPC (dipalmitoylphosphatidylcholine), POPC (palmitoyloleoylphosphatidylcholine) and distearoyl-phosphatidylcholine; fatty acid esters; glycerol esters; sphingolipids; cardiolipin; cerebrosides; and ceramides; and mixtures thereof. Neutral lipids also include cholesterol and other 3 $\beta$ OH-sterols.

Other lipids contemplated herein, include: phosphatidylglycerol; phosphatidylglycerols, such as DOPG (dioleoylphosphatidylglycerol), DPPG (dipalmitoylphosphatidylglycerol), and distearoyl-phosphatidylglycerol; phosphatidylserine; phosphatidylserines, such as dioleoyl- or dipalmitoylphosphatidylserine and diphosphatidylglycerols.

Examples of cationic lipid compounds include, but are not limited to: Lipofectin (Life Technologies, Inc., Burlington, Ont.)(1:1 (w/w) formulation of the cationic lipid N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) and dioleoylphosphatidylethanol-amine (DOPE)); LipofectAMINE (Life Technologies, Burlington, Ont., see U.S. Patent No. 5,334,761) (3:1 (w/w) formulation of polycationic lipid 2,3-dioleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate (DOSPA) and dioleoylphosphatidyl-ethanolamine (DOPE)), LipofectAMINE PLUS (Life Technologies, Burlington, Ont. see U.S. Patent Nos. 5,334,761 and 5,736,392; see, also U.S. Patent No. 6,051,429) (LipofectAmine and Plus reagent), LipofectAMINE 2000 (Life Technologies, Burlington, Ont.; see also International PCT application No. WO 00/27795) (Cationic lipid), Effectene (Qiagen, Inc., Mississauga, Ontario) (Non liposomal lipid formulation), Metafectene (Biontex, Munich, Germany) (Polycationic lipid), Eu-fectins (Promega Biosciences, Inc., San Luis Obispo, CA) (ethanolic cationic lipids numbers 1 through 12: C<sub>52</sub>H<sub>106</sub>N<sub>6</sub>O<sub>4</sub>.4CF<sub>3</sub>CO<sub>2</sub>H, C<sub>88</sub>H<sub>178</sub>N<sub>8</sub>O<sub>4</sub>S<sub>2</sub>.4CF<sub>3</sub>CO<sub>2</sub>H, C<sub>40</sub>H<sub>84</sub>NO<sub>3</sub>P.CF<sub>3</sub>CO<sub>2</sub>H, C<sub>50</sub>H<sub>103</sub>N<sub>7</sub>O<sub>3</sub>.4CF<sub>3</sub>CO<sub>2</sub>H, C<sub>55</sub>H<sub>116</sub>N<sub>8</sub>O<sub>2</sub>.6CF<sub>3</sub>CO<sub>2</sub>H, C<sub>49</sub>H<sub>102</sub>N<sub>6</sub>O<sub>3</sub>.4CF<sub>3</sub>CO<sub>2</sub>H, C<sub>44</sub>H<sub>89</sub>N<sub>5</sub>O<sub>3</sub>.2CF<sub>3</sub>CO<sub>2</sub>H, C<sub>100</sub>H<sub>206</sub>N<sub>12</sub>O<sub>4</sub>S<sub>2</sub>.8CF<sub>3</sub>CO<sub>2</sub>H, C<sub>162</sub>H<sub>330</sub>N<sub>22</sub>O<sub>9</sub>.13CF<sub>3</sub>CO<sub>2</sub>H, C<sub>43</sub>H<sub>88</sub>N<sub>4</sub>O<sub>2</sub>.2CF<sub>3</sub>CO<sub>2</sub>H, C<sub>43</sub>H<sub>88</sub>N<sub>4</sub>O<sub>3</sub>.2CF<sub>3</sub>CO<sub>2</sub>H, C<sub>41</sub>H<sub>78</sub>NO<sub>8</sub>P); Cytofectene (Bio-Rad, Hercules, CA ) (mixture of a cationic lipid and a neutral lipid), GenePORTER (Gene Therapy Systems Inc., San Diego, CA) (formulation of a neutral lipid (Dope) and a cationic lipid) and FuGENE 6 (Roche Molecular Biochemicals, Indianapolis, IN) (Multi-component lipid based non-liposomal reagent).

#### (ii) Non-lipid cationic compounds

Non-lipid cationic reagents include, but are not limited to SUPERFECT $\beta$  (Qiagen, Inc., Mississauga, ON) (Activated dendrimer (cationic polymer:charged amino groups) and CLONfectin $\beta$  (Cationic amphiphile N-t-butyl-N'-tetradecyl-3-tetradecyl-aminopropionamide) (Clontech, Palo Alto, CA).

Pyridinium amphiphiles are double-chained pyridinium compounds, which are essentially nontoxic toward cells and exhibit little cellular preference for the ability to transfect cells. Examples of a pyridinium amphiphiles are the pyridinium chloride surfactants such as SAINT-2 (1-methyl-4-(1-octadec-9-enyl-nonadec-10-enylenyl) pyridinium chloride) (see, e.g., van der Woude *et al.* (1997) *Proc. Natl. Acad. Sci. U.S.A.* 94:1160). The pyridinium chloride surfactants are typically mixed with neutral helper lipid compounds, such as dioleoylphosphatidylethanolamine (DOPE), in a 1:1 molar ratio. Other Saint

derivatives of different chain lengths, state of saturation and head groups can be made by those of skill in the art and are within the scope of the present methods.

(b) Energy

Delivery agents also include treatment or exposure of the cell and/or nucleic acid molecules, but generally the cells, to sources of energy, such as sound and electrical energy.

(i) Ultrasound

For *in vitro* and *in vivo* transfection, the ultrasound source should be capable of providing frequency and energy outputs suitable for promoting transfection. For example, the output device can generate ultrasound energy in the frequency range of 20 kHz to about 1 MHz. The power of the ultrasound energy can be, for example, in the range from about 0.05 w/cm<sup>2</sup> to 2 w/cm<sup>2</sup>, or from about 0.1 w/cm<sup>2</sup> to about 1 w/cm<sup>2</sup>. The ultrasound can be administered in one continuous pulse or can be administered as two or more intermittent pulses, which can be the same or can vary in time and intensity.

Ultrasound energy can be applied to the body locally or ultrasound-based extracorporeal shock wave lithotripsy can be used for "in-depth" application. The ultrasound energy can be applied to the body of a subject using various ultrasound devices. In general, ultrasound can be administered by direct contact using standard or specially made ultrasound imaging probes or ultrasound needles with or without the use of other medical devices, such as scopes, catheters and surgical tools, or through ultrasound baths with the tissue or organ partially or completely surrounded by a fluid medium. The source of ultrasound can be external to the subject 's body, such as an ultrasound probe applied to the subject 's skin which projects the ultrasound into the subject 's body, or internal, such as a catheter having an ultrasound transducer which is placed inside the subject 's body. Suitable ultrasound systems are known (see, e.g., International PCT application No. WO 99/21584 and U.S. Patent No. 5,676,151).

When the cationic compound and nucleic acid molecules, such as DNA, are administered systemically, the ultrasound can be applied to one or several organs or tissues simultaneously to promote nucleic acid molecule delivery to multiple areas of the subject 's body. Alternatively, the ultrasound can be applied selectively to specific areas or tissues to promote selective uptake of the nucleic acid molecules, such as DNA.

The transfection efficiency of the ultrasound can also be enhanced by using contrast reagents, which serve as artificial cavitation nuclei, such as Albunex (Molecular Biosystems, San Diego, CA), Imagent (Alliance Pharmaceutical, San Diego, CA), Levovist-SHU

(Schering AG, Berlin, Germany), Definity (E.I. du Pont de Nemour, Wilmington, DL), STUC (Washington University, St Louis, MO) and the introduction of gaseous microbubbles. A contrast reagent can be introduced locally, such as a joint; introduced systematically, with the enhancement of cavitation efficiency by focusing lithotripter shock waves at a defined area; 5 or by targeting a contrast reagent to a particular site and then enhancing cavitation efficiency by focusing lithotripter shock waves.

**(ii) Electroporation**

Electroporation temporarily opens up pores in a cell 's outer membrane by use of pulsed rotating electric fields. Methods and apparatus used for electroporation *in vitro* and *in vivo* are well known (see, e.g., U.S. Patent Nos. 6,027,488, 5,993,434, 5,944,710, 5,507,724, 10 5,501,662, 5,389,069, 5,318,515). Standard protocols may be employed.

**E. IN VITRO AND EX VIVO DELIVERY OF NUCLEIC ACIDS TO CELLS**

**1. In vitro Delivery**

Nucleic acid molecules, such as DNA, can be added to synchronized cells *in vitro* either separately or mixed with one or more delivery agents and with or without the 15 application of ultrasound or electrical energy. In general, if energy is applied, it is applied prior to contacting the cells with the nucleic acid molecule.

In general, nucleic acid molecules, such as DNA, mixed with cationic lipids/compounds can be added to synchronized cells as described in the EXAMPLES. 20 Parameters important for optimization of the delivery of nucleic acid molecules, such as DNA, into target cells will be apparent to those of skill in this art. These parameters include, for example, the cell cycle phase during which nucleic acid molecule is added, the delivery method, the nucleic acid molecules, such as DNA, the concentration of nucleic acid molecules, the cell growth medium, the cell culture conditions, the length of time cells are 25 exposed to the nucleic acid, the toxicity of the delivery method to the target cell type, and the amount and time of use of ultrasound or electroporation among other parameters. It may be necessary to optimize these parameters for different nucleic acid molecules, such as DNA, and target cell types. Such optimization is routine employing the guidance provided herein. In addition, the rapid screening method can provide direction as to what parameters may need 30 to be adjusted to optimize delivery (see EXAMPLES). Alteration of culture conditions, time, reagent concentrations and other parameters, for use with different combinations of cationic compounds and target cell types and to optimize delivery, can be empirically determined. If ultrasound energy is required to be used to enhance transfection efficiency, it can be applied

as described below and in the EXAMPLES. Electroporation can be performed as described below or by any suitable protocol known to those of skill in this art.

The contacting of cells with cationic compounds and nucleic acid molecules, such as DNA, in separate and distinct steps can be generally carried out as described in the EXAMPLES. Those of skill in the art can readily vary the order of the application of the components to the target cell based on the disclosure herein.

## 2. *Ex vivo* Gene Therapy

Delivery of nucleic acid molecules, such as DNA, is carried out as described above in *in vitro* delivery. After selection has been completed, cells harboring the nucleic acid molecules, such as DNA, are introduced into the subject target by a variety of means, including injection, such as subcutaneous, intramuscular, intraperitoneal, intravascular and intralymphatic and intra-articular injection. The cells can be administered with or without the aid of medical devices such as arthroscopes, other scopes or various types of catheters.

## 3. Gene Therapy in Connective Tissue and Rheumatic Diseases

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by joint inflammation and progressive cartilage and bone destruction. Treatment of RA is problematic with current strategies since relatively high systemic doses are necessary to achieve therapeutic levels of anti-rheumatic drugs in the joints. In addition, the available treatments are associated with significant untoward side effects. Gene therapy is thus a more efficient system for delivery of therapeutic molecules to the site of inflammation in the treatment of connective tissue diseases, rheumatic diseases and chronic erosive joint diseases such as RA, osteoarthritis, ankylosing spondylitis and juvenile chronic arthritis.

In a diarthrodial movable joint, smooth articulation is ensured by the macromolecular structure of the articular cartilage which covers the ends of the bones. The cavity or joint space that occurs at the location of adjacent bones is lined by a tissue referred to as the synovium. The synovium contains macrophage-like type A cells (presumably derived from macrophage/monocyte precursors and exhibiting phagocytic activity) and fibroblast-like type B cells (more fibroblast in appearance and associated with production of hyaluronic acid and other components of the joint fluid). Underlying the synovium is a sparsely cellular subsynovium which may be fibrous, adipose or areolar in nature. Fibroblast-like synoviocytes (FLS) are distinguishable from normal fibroblast cells in the subintimal synovium by differential gene expression patterns. FLS have been shown to express high levels of uridine diphosphoglucose dehydrogenase (UDPGD), high levels of vascular cell

adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) as well as CD44 (hyaluronic acid receptor), fibronectin receptor and  $\beta$ 3 integrins. Sublining fibroblasts or fibroblasts from other sources do not express these markers or express them at lower levels [see, e.g., Edwards (1995) *Ann. Rheum. Dis.* 54:395-397; Firestein (1996) *Arthritis Rheum.*

5 39:1781-1790; Edwards (2000) *Arthritis Res.* 2:344-347].

Disease progression in RA involves the thickening of the synovial lining due to the proliferation of fibroblast-like synoviocytes (FLS) and infiltration by inflammatory cells (e.g., lymphocytes, macrophages and mast cells). The normal biology of synoviocytes is also altered in the pathological process of RA, including invasion and destruction of articular

10 cartilage and bone. In addition to the production of elastase and collagenase, synoviocytes mediate the pathophysiological process of RA by expression of cell surface proteins involved in the recruitment and activation of lymphocytes and macrophages within the synovium.

Proliferation of synovial cells leads to a pannus tissue that invades and overgrows cartilage, leading to bone destruction and destruction of joint structure and function. Proinflammatory 15 cytokines, for example, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-1 (IL-1) play key roles in inflammation and joint damage associated with RA. Pathological effects caused by these cytokines include leukocytic infiltration leading to synovial hyperplasia, cell activation, cartilage breakdown and inhibition of cartilage matrix synthesis.

Nucleic acid transfer to rheumatoid synovial tissue may result in the production of 20 mediators that inhibit inflammation or hyperplasia or provide toxic substances that specifically destroy the diseased synovium. Retroviral delivery of nucleic acid encoding interleukin-1 receptor antagonist (IL1-RA) *ex vivo* and transduction of synoviocytes has been used in gene therapy of RA in humans to inhibit inflammation [see, e.g., Evans (1996) *Human Gen. Ther.* 7:1261-1280 and Del Vecchio *et al.* (2001) *Arthritis Res.* 3:259-263]. 25 Adenoviral vectors have been proposed for delivery of nucleic acid encoding an IL-1 receptor antagonist to synoviocytes in *in vivo* transduction methods [see, e.g., U.S. Patent No. 5,747,072 and PCT Application Publication No. WO 00/52186].

Artificial chromosomes provide advantages over virus-based systems for gene 30 therapy. For example, artificial chromosome expression systems (ACes), and other artificial chromosomes as described in U.S. Patent Nos. 6,025,155 and 6,077,697 and PCT Application No. WO97/40183, serve as non-integrating, non-viral vectors with a large capacity for delivering large nucleic acids and/or multiple copies of a particular nucleotide sequence into cells, such as synoviocytes, both *in vitro* and *in vivo*. Such artificial chromosome systems

offer further advantages in that they allow stable and predictable expression of genes producing single or multiple proteins over long periods of time.

The methods provided herein may be used to introduce large nucleic acids, such as, for example, artificial chromosomes, into primary cells, such as, for example, synoviocytes (e.g., fibroblast-like synoviocytes) and skin fibroblasts, and skeletal muscle fibroblast cell lines. Thus, included among the methods provided herein is a method for introducing heterologous nucleic acid into a synoviocyte by introducing in a chromosome, such as for example an artificial chromosome, into the synoviocyte. In one such embodiment, the artificial chromosome is an ACes. The synoviocyte can be, for example, a fibroblast-like synoviocyte.

A particular method provided herein for introducing a large nucleic acid molecule into a synoviocyte includes steps of obtaining a synoviocyte that is in a pre-selected phase and contacting the synoviocyte with the nucleic acid molecule. In one method, the nucleic acid molecule is contacted with a synchronous population of synoviocytes in which the population has been synchronized to be in a pre-selected phase at the time of contact.

Such methods can further include steps of exposing the nucleic acid molecule to a delivery agent and contacting the synoviocyte with the nucleic acid molecule. In a particular embodiment of this method, the delivery agent is not energy. In one embodiment, the large nucleic acid molecule is a chromosome. For example, the nucleic acid can be an artificial chromosome, such as an ACes. In a particular embodiment, the synoviocyte is a fibroblast-like synoviocyte. Any delivery agents, such as described herein, may be used in such methods. For example, the delivery agent can be one that includes a cationic compound.

Also provided are methods for introducing a large nucleic acid molecule into a synoviocyte that include a step of obtaining a synoviocyte that is in a pre-selected phase and contacting the synoviocyte with the nucleic acid molecule. Such methods can further include steps of exposing the nucleic acid molecule to a delivery agent, exposing the synoviocyte to a delivery agent and contacting the synoviocyte with the nucleic acid molecule, whereby the nucleic acid molecule is delivered into the synoviocyte, and wherein the steps are performed sequentially in any order or simultaneously. In some embodiments of the method, if the delivery agent is energy, it is not applied to the nucleic acid molecule and it is not applied to the synoviocyte after contacting the synoviocyte with the nucleic acid molecule. The nucleic acid may be any nucleic acid. In particular embodiments, the nucleic acid is a large nucleic acid, chromosome, artificial chromosome or ACes. In a further particular embodiment, the

synoviocyte is a fibroblast-like synoviocyte. Delivery agents, such as described herein, may be used in such methods. For example, the delivery agent can be one that includes a cationic compound.

Another method for delivering a nucleic acid molecule into a synoviocyte provided  
5 herein includes steps of contacting the synoviocyte in the presence or absence of the nucleic acid molecule with a delivery agent, and applying ultrasound energy or electrical energy to the synoviocyte, wherein the contacting and applying are performed sequentially or simultaneously, and then contacting the synoviocyte with the nucleic acid molecule, whereby the nucleic acid molecule is delivered into the synoviocyte. The nucleic acid may be any  
10 nucleic acid. In particular embodiments, the nucleic acid is a large nucleic acid, chromosome, artificial chromosome or ACes. In a further particular embodiment, the synoviocyte is a fibroblast-like synoviocyte. Numerous delivery agents, including agents such as those described herein, may be used in such methods. For example, the delivery agent can be one that includes a cationic compound. In one embodiment, the energy is  
15 ultrasound.

Thus, provided herein are methods of delivering nucleic acids, in particular, large nucleic acids, such as chromosomes, including artificial chromosomes, e.g., ACes, into primary cells, including synoviocytes and fibroblasts. These methods may be used *in vitro* and *in vivo*.

20 Also provided herein is a synoviocyte in a pre-selected phase comprising a large heterologous nucleic acid, a heterologous chromosome or portion thereof, or an artificial chromosome. In one embodiment, the artificial chromosome is an ACes. Such synoviocytes include fibroblast-like synoviocytes. The synoviocytes may be from any species, including, but not limited to mammalian species. For example, synoviocytes containing large nucleic  
25 acids, such as for example artificial chromosomes (e.g., ACes) include primate synoviocytes, as well as rodent, rabbit, monkey, dog, horse and human synoviocytes.

The ability to achieve delivery of large nucleic acids into such cells demonstrates the usefulness of the methods in gene therapy applications as well as in the testing in animal models of disease of possible therapeutic molecules for use in gene therapy methods. Thus,  
30 provided herein are methods of treating diseases or modulating disease processes which include steps of introducing a large nucleic acid molecule, chromosome or portion thereof, or artificial chromosome into a subject who has the disease.

A method for treating or modulating a rheumatic disease process in a subject is provided herein. In one embodiment, the method includes steps of introducing a large nucleic acid into the subject, wherein the large nucleic acid contains nucleic acid that is or that encodes an agent that modulates a rheumatic disease process. For example, the nucleic acid can be or can encode a molecule that has an anti-rheumatic effect. Processes associated with rheumatic diseases are known in the art and are described herein. For example, one such process is an inflammatory process that includes processes of cell activation, infiltration, proliferation and recruitment. In a particular embodiment of this method, the disease is rheumatoid arthritis. The nucleic acid may be, for example, a chromosome or portion thereof or an artificial chromosome, e.g., an ACes. In particular embodiments, the large nucleic acid is introduced into a site of inflammation in the subject. One possible site of inflammation is a joint.

Also provided is a method for treating a rheumatic disease in a subject in which a large nucleic acid is introduced into the subject, wherein the large nucleic acid contains nucleic acid that is or that encodes a therapeutic agent. For example, the nucleic acid can be or can encode a molecule that has an anti-rheumatic effect. In a particular embodiment of this method, the disease is rheumatoid arthritis. The nucleic acid may be, for example, a chromosome or portion thereof or an artificial chromosome, e.g., an ACes. In an embodiment of this method, the large nucleic acid is introduced into a site of inflammation in the subject. One possible site of inflammation is a joint.

In the methods for modulating a rheumatic disease process or treating a rheumatic disease, the method may be practiced in any format, including *ex vivo* and *in vivo* formats. Thus, for example, the nucleic acid can be introduced into a cell *in vitro* and then transferred into the subject. Alternatively, the nucleic acid can be introduced into a cell *in vivo*. In a particular embodiment, the nucleic acid is introduced into a synoviocyte, which can be, for example, a fibroblast-like synoviocyte. The nucleic acid that is introduced can comprise any nucleic acid that is or that encodes a molecule that has an anti-rheumatic effect in the subject. For example, the molecule may alter, counteract or diminish a process of the disease. The molecule may ameliorate symptoms of the disease. Molecules that provide anti-rheumatic effects in subjects with RA are known in the art [see, e.g., Vervoordeldonk and Tak (2001) *Best Prac. Res. Clin. Rheumatol.* 15:771-788 and WO 00/52186]. Such molecules include anti-inflammatory or immunomodulatory molecules. For example, interleukin-1 receptor antagonists, soluble interleukin-1 receptor, soluble tumor necrosis factor receptor, interferon-

$\beta$ , interleukin-4, interleukin-10, interleukin-13, transforming growth factor  $\beta$ , dominant negative IkappaB-kinase, FasL, Fas-associated death domain protein or CTLA-4 are among molecules that can have anti-rheumatic effects.

Also provided is a method of identifying, evaluating or testing a nucleic acid as a

5 potential therapeutic agent in the treatment of a connective tissue or rheumatic disease by introducing into an animal model of a connective tissue or rheumatic disease a large nucleic acid molecule. The nucleic acid molecule can be one that includes nucleic acid that is or encodes a candidate therapeutic agent. The method may include a step of determining if the nucleic acid molecule has any effects, and in particular any anti-rheumatic effects, on the  
10 animal. For example, in determining if the nucleic acid molecule has any effects on the animal, it may be evaluated whether one or more conditions of the disease is effected, such as, for example, amelioration of or reduction in an adverse condition. In a particular embodiment, the disease is a rheumatic disease, such as, for example rheumatoid arthritis. The animal is any animal in which the disease may be modeled. For example, the animal  
15 may be a mammal. In particular embodiments the animal is a monkey, rodent, rabbit, dog, cat, horse, cow, pig or primate. The large nucleic acid may be, for example, a chromosome, or portion thereof, or an artificial chromosome, for example, an ACes. In particular embodiments, the nucleic acid molecule is in a synoviocyte, such as, for example, a fibroblast-like synoviocyte. In further embodiments, the nucleic acid is introduced into a  
20 joint of the animal. The nucleic acid molecule may be introduced into the animal using *in vitro* or *in vivo* formats. For example, the nucleic acid can be introduced into a cell *in vitro* and then be transferred into the animal. In another embodiment, the nucleic acid is introduced into a cell *in vivo*.

Animal models include, for example, animal models of RA. Several animal models

25 of RA, and methods for generating such models, are known in the art. Such models include adjuvant-induced arthritis (AA) [see, e.g., Kong *et al.* (1999) *Nature* 4023:304-309] and collagen type II-induced arthritis [see, e.g., Tak *et al.* (1999) *Rheumatology* 38:362-369; Han *et al.* (1998) *Autoimmunity* 28:197-208; Gerlag *et al.* (2000) *J. Immunology* 165:1652-1658]. For example, experimental induction of adjuvant-induced arthritis in Lewis rats leads to  
30 severe inflammation in the bone marrow and soft tissues surrounding joints accompanied by extensive local bone and cartilage destruction, loss of bone mineral density and crippling [see, e.g., Bendele *et al.* (1999) *Arthritis Rheum.* 42:498-506].

The following examples are included for illustrative purposes only and are not intended to limit the scope of the invention.

## EXAMPLE 1

### Preparation of Artificial Chromosomes

#### A. GFP Chromosome contained in A9 cell line Plasmids

Plasmid pIRES-EGFP (see SEQ ID No. 13, plasmid obtained from Clontech, CA, and is well known, see, e.g., U.S. Patent Nos. 6,034,228, 6,037,133, 5,985,577, 5,976,849, 5,965,396, 5,976,796, 5,843,884, 5,962,265, 5,965,396; see, also, U.S. Patent No. 4,937,190).

This plasmid contains the internal ribosome entry site (IRES; Jackson (1990) *Trends Biochem.* 15:477-483; Jang *et al.* (1988) *J. Virol.* 62:2636-2643) of the encephalomyocarditis virus (ECMV) between the MCS and the enhanced green fluorescent protein (EGFP) coding region. This permits the gene of interest (cloned into the MCS) and the EGFP gene to be translated from a single bicistronic mRNA transcript. Plasmid pIRES2-EGFP is designed for selection, by flow cytometry and other methods, of transiently transfected mammalian cells that express EGFP and the protein of interest. This vector can also be used to express EGFP alone or to obtain stably transfected cell lines without drug and clonal selection.

Enhanced GFP (EGFP) is a mutant of GFP with a 35-fold increase in fluorescence.

This variant has mutations of Ser to Thr at amino acid 65 and Phe to Leu at position 64 and is encoded by a gene with optimized human codons (see, e.g., U.S. Patent No. 6,054,312). EGFP is a red-shifted variant of wild-type GFP (Yang *et al.* (1996) *Nucl. Acids Res.* 24:4592-4593; Haas *et al.* (1996) *Curr. Biol.* 6:315-324; Jackson *et al.* (1990) *Trends Biochem.* 15:477-483) that has been optimized for brighter fluorescence and higher expression in mammalian cells (excitation maximum = 488 nm; emission maximum = 507 nm). EGFP encodes the GFPmut1 variant (Jackson (1990) *Trends Biochem.* 15:477-483) which contains the double-amino-acid substitution of Phe-64 to Leu and Ser-65 to Thr. The coding sequence of the EGFP gene contains more than 190 silent base changes which correspond to human codon-usage preferences (Jang *et al.* (1988) *J. Virol.* 62:2636-2643). Sequences flanking EGFP have been converted to a Kozak consensus translation initiation site (Huang *et al.* (1990) *Nucleic Acids Res.* 18: 937-947) to further increase the translation efficiency in eukaryotic cells.

Plasmid pIRES-EGFP was derived from PIRESneo (originally called pCIN4) by replacing the neo gene downstream of the IRES sequence with the EGFP coding region. The

IRES sequence permits translation of two open reading frames from one mRNA transcript. The expression cassette of pIRES-EGFP contains the human cytomegalovirus (CMV) major immediate early promoter/enhancer followed by a multiple cloning site (MCS), a synthetic intron (IVS; Huang *et al.* (1990) *Nucleic Acids Res.* 18: 937-947), the EMCV IRES followed 5 by the EGFP coding region and the polyadenylation signal of bovine growth hormone.

Location of Features (with reference to SEQ ID No. 13):

Human cytomegalovirus (CMV) immediate early promoter: 232-820;  
10 MCS 909-974;  
IVS 974-1269;  
IRES of ECMV 1299-1884;  
Enhanced green fluorescent protein (EGFP) gene 1905-2621;  
fragment containing the bovine polyA signal 2636-2913;  
Col E1 origin of replication 3343-4016; and  
15 Ampicillin resistance gene 5026-4168

Propagation in *E. coli*

Suitable host strains: DH5a, HB101, and other general purpose strains. Single-stranded DNA  
20 production requires a host containing an F plasmid such as JM101 or XL1-Blue.  
Selectable marker: plasmid confers resistance to kanamycin (30 µg/ml) to *E. coli* hosts.  
*E. coli* replication origin: pUC  
Copy number: ~500  
Plasmid incompatibility group: pMB1/ColE1

25 **pCHEGFP2**

Plasmid pCHEGFP2 was constructed by deletion of the Nsi1/SmaI fragment from  
pIRES-EGFP. Plasmid pIRES-EGFP contains the coding sequence for a 2.1 kB Nru 1/Xho  
fragment of pCHEGFP2 containing the CMV promoter, synthetic intron, EGFP coding  
sequence and bovine growth hormone polyadenylation signal. Digestion of pIRES-EGFP  
30 with Nru 1 and Sma 1, yielded a 2.1 kb fragment. Digested DNA was fractionated by agarose  
gel electrophoresis, the separated band was excised and then eluted from the gel using the  
Qiaex 11 gel purification system (Qiagen, Mississauga, Ontario).

**pFK161**

Cosmid pFK161 was obtained from Dr. Gyula Hadlaczky and contains a 9 kb *NotI* insert derived from a murine rDNA repeat (see clone 161 described in PCT Application Publication No. WO97/40183 by Hadlaczky *et al.* for a description of this cosmid). This 5 cosmid, referred to as clone 161 contains sequence corresponding to nucleotides 10,232- 15,000 in SEQ ID NO. 16. It was produced by inserting fragments of the megachromosome (see, U.S. Patent No. 6,077,697 and International PCT application No. (WO 97/40183); for example, H1D3, which was deposited at the European Collection of Animal Cell Culture (ECACC) under Accession No. 96040929, is a mouse-hamster hybrid cell line carrying this 10 megachromosome) into plasmid pWE15 (Stratagene, La Jolla, California) as follows. Half of a 100 µl low melting point agarose block (mega-plug) containing isolated SATACs was digested with *NotI* overnight at 37°C. Plasmid pWE15 was similarly digested with *NotI* overnight. The mega-plug was then melted and mixed with the digested plasmid, ligation 15 buffer and T4 ligase. Ligation was conducted at 16°C overnight. Bacterial DH5 $\alpha$  cells were transformed with the ligation product and transformed cells were plated onto LB/Amp plates. Fifteen to twenty colonies were grown on each plate for a total of 189 colonies. Plasmid DNA was isolated from colonies that survived growth on LB/Amp medium and was analyzed by Southern blot hybridization for the presence of DNA that hybridized to a pUC19 probe. This screening methodology assured that all clones, even clones lacking an insert but 20 yet containing the pWE15 plasmid, would be detected.

Liquid cultures of all 189 transformants were used to generate cosmid minipreps for analysis of restriction sites within the insert DNA. Six of the original 189 cosmid clones contained an insert. These clones were designated as follows: 28 (~9-kb insert), 30 (~9-kb insert), 60 (~4-kb insert), 113 (~9-kb insert), 157 (~9-kb insert) and 161 (~9-kb insert). 25 Restriction enzyme analysis indicated that three of the clones (113, 157 and 161) contained the same insert.

For sequence analysis the insert of cosmid clone no. 161 was subcloned as follows. To obtain the end fragments of the insert of clone no. 161, the clone was digested with *NotI* and *BamHI* and ligated with *NotI/BamHI*-digested pBluescript KS (Stratagene, La Jolla, 30 California). Two fragments of the insert of clone no. 161 were obtained: a 0.2-kb and a 0.7-kb insert fragment. To subclone the internal fragment of the insert of clone no. 161, the same digest was ligated with *BamHI*-digested pUC19. Three fragments of the insert of clone no. 161 were obtained: a 0.6-kb, a 1.8-kb and a 4.8-kb insert fragment.

The insert corresponds to an internal section of the mouse ribosomal RNA gene (rDNA) repeat unit between positions 7551-15670 as set forth in GENBANK accession no. X82564, which is provided as SEQ ID NO. 5. The sequence data obtained for the insert of clone no. 161 is set forth in SEQ ID NOS. 6-12. Specifically, the individual subclones 5 corresponded to the following positions in GENBANK accession no. X82564 (i.e., SEQ ID NO. 5) and in SEQ ID NOS. 6-12:

Subclone	Start	End	Site	SEQ ID No.
in X82564				
161k1	7579	7755	<i>NotI, BamHI</i>	6
161m5	7756	8494	<i>BamHI</i>	7
161m7	8495	10231	<i>BamHI</i>	8(shows only sequence corresponding to nt. 495-8950), 9 (shows only sequence corresponding to nt. 9851- 10231)
161m12	10232	15000	<i>BamHI</i>	10 (shows only sequence corresponding to nt. 10232-10600), 11 (shows only sequence corresponding to nt. 14267-15000)
161k2	15001	15676	<i>NotI, BamHI</i>	12

10 The sequence set forth in SEQ ID NOS. 6-12 diverges in some positions from the sequence presented in positions 7551-15670 of GENBANK accession no. X82564. Such divergence may be attributable to random mutations between repeat units of rDNA.

For use herein, the rDNA insert from the clone was prepared by digesting the cosmid with *NotI* and *BglII* and was purified as described above. Growth and maintenance of bacterial stocks and purification of plasmids were performed using standard well known methods (see, e.g., Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual*, 2<sup>nd</sup> Edition, Cold Spring Harbor Laboratory Press), and plasmids were purified from bacterial cultures using Midi - and Maxi-preps Kits (Qiagen, Mississauga, Ontario).

## B. PREPARATION OF THE GFP, MURINE A9 CELL LINE

### Cell culture and transfection

20 The murine A9 cell line was obtained from ATCC and cells were thawed and maintained as described below. Briefly, cells were plated at a density of  $2 \times 10^6$  cells per 15 cm tissue culture dish (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ) in growth medium containing of 90% DMEM (Canadian Life Technologies Burlington, ON) and 10% FBS (Can Sera, Rexdale ON), and were maintained at 37°C, 5% CO<sub>2</sub>. Cultures were 25 routinely passaged when cells reached 70%-80% confluence. Sub culturing was carried out as follows: medium was removed by aspiration, 10 ml of 1X trypsin-EDTA (Canadian Life

Technologies Burlington, ON) was dispensed onto the cell monolayer and the dish gently swirled to distribute the trypsin-EDTA. Finally, the bulk of the trypsin-EDTA was removed by aspiration, and the dish placed at 37°C for 5 minutes. To quench the trypsin-EDTA, 10 ml of growth medium was added to the dish, and the single cell suspension was transferred to a 5 50 ml conical tube. Cell counts were performed using a cell counting apparatus (Beckman-Coulter, Hialeah FL). The cells were diluted and re-plated as described above. For cryo-storage, cultures were harvested by treatment with trypsin-EDTA, counted and the cell suspension then centrifuged at 500Xg for 5 minutes in a swinging bucket centrifuge. The cell pellet was resuspended in freezing medium containing 90% DMEM, 20% FBS and 10% 10 DMSO (Sigma-Aldrich, Oakville, ON) at a density of  $1 \times 10^7$  cells/ml. One ml aliquots of the cell suspension were then dispensed into cryo-vials (Nunc, Rochester NY), frozen over night in an isopropanol filled container (NUNC, Rochester NY) and placed at -70°C and then transferred to the gas phase of a liquid nitrogen freezer for long-term storage.

A9 cells were transfected using the Ca<sub>2</sub>PO<sub>4</sub> co-precipitation method (see, e.g.,

15 Graham *et al.* (1978) *Virology* 52:456-457; Wigler *et al.* (1979) *Proc. Natl. Acad. Sci. U.S.A.* 76:1373-1376; and (1990) *Current Protocols in Molecular Biology*, Vol. 1, Wiley Inter-Science, Supplement 14, Unit 9.1.1-9.1.9). One day prior to transfection, A9 cells were plated at a density of  $2 \times 10^6$  cells per 10 cm dish and 3 hours before transfection the medium was replaced with fresh growth medium. 140 µg of the 9 kb rDNA, *NotI* and 5 µg of the 2.1 20 kB CMV-EGFP *Xhol/Nrul* fragments were mixed, co-precipitated and used to prepare the Ca<sub>2</sub>PO<sub>4</sub> co-precipitate (Calcium Phosphate Transfection System, (Canadian Life Technologies Burlington, ON) which was distributed onto 2 10-cm dishes of subconfluent A9 cells. The DNA-Ca<sub>2</sub>PO<sub>4</sub> complexes were left on the cells for 18 hours, after which the precipitate was removed by aspiration and cells were subjected to glycerol shock for 1.5 25 minutes. After glycerol shock, the cell monolayers were gently washed with 2 X 10 ml of dPBS (Canadian Life Technologies Burlington, ON), followed by addition of 10 ml pre-warmed growth medium. Finally dishes were returned to the incubator and were maintained at 37°C, 5% CO<sub>2</sub>. After 3 hours recovery, each dish was passaged onto 3X 15 cm tissue dishes

30 GFP fluorescence of cultures was monitored visually during culture using an inverted microscope equipped with epifluorescence illumination (Axiovert 25; Zeiss, (North York ON) and #41017 Endow GFP filter set (Chroma Technologies, Brattleboro, VT). Enrichment of GFP expressing populations was carried out as described below.

**Enrichment of GFP expressing cell populations by  
Fluorescence Activated Cell Sorting**

Cell sorting was carried out using a FACS Vantage flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) equipped with turbo-sort option and 2 Innova 306 lasers (Coherent, Palo Alto CA). For cell sorting a 70 µm nozzle was used. The sheath buffer was changed to PBS (maintained at 20 p.s.i.). GFP was excited with a 488 nm Laser beam and excitation detected in FL1 using a 500 EFLP filter. Forward and side scattering was adjusted to select for viable cells. Only viable cells were then analyzed for GFP fluorescence. Gating parameters were adjusted using wild type A9 cells as negative control and GFP CHO cells as positive control.

For the first round of sorting, A9 cells were harvested 4 days post-transfection, resuspended in 10 ml of growth medium and sorted for GFP expressing populations using parameters described above. GFP positive cells were dispensed into a volume of 5-10 ml of growth medium supplemented with 1X penicillin/streptomycin (Canadian Life Technologies Burlington, ON) while non-expressing cells were directed to waste. The expressing cells were further diluted to 50 ml using the same medium, plated onto 2X15 cm dishes and cultured as described in the previous section. When the sorted populations reached confluence they were resorted to enrich for GFP expressing cells. A total of 4 sequential sorts were carried out, achieving enrichments of as high as 89% GFP expressing cells after the final sort. The final GFP expressing populations were expanded for cryo-preservation and for fluorescence in-situ hybridization screening (see below). Single cell clones were established from populations of interest by using the flow cytometer to direct GFP expressing single cells to individual wells of 96 well plates. These were cultured as described above.

**Fluorescence In-Situ Hybridization**

Fluorescence In-Situ Hybridization (FISH) screening was carried out on GFP enriched populations and single cell clones to detect amplification and/or artificial chromosome formation. Preparation of metaphase spreads and hybridizations were performed (see, Telenius *et al.* (1999) *Chromosome Res* 7:3-7). Probes used include pSAT 1, which recognizes the mouse major repeat (see, e.g., Wong *et al.* (1988) *Nucl. Acids Res.* 16:11645-11661), pFK161, which hybridizes to the mouse rDNA-containing regions and a PCR generated probe against the mouse minor repeat.

Thus, in one method provided herein for generating an artificial chromosome, such as an ACes, heterologous nucleic acid that includes a selectable marker, e.g., nucleic acid

encoding a fluorescent protein or other protein that may be readily detected using flow cytometry-based methods or other methods, including, for example, fluorimetry, cell imaging or fluorescence spectroscopy, is introduced into a cell. For example, rDNA and DNA encoding enhanced green fluorescent protein (EGFP) may be introduced into cells, e.g., A9 cells. The transfected cells may be selected on the basis of properties detectable by flow cytometry-based methods, or other methods, including, for example, fluorimetry, cell imaging or fluorescence spectroscopy, e.g., fluorescent properties. For example, cells containing a fluorescent protein may be isolated from nontransfected cells using a fluorescence-activated cell sorter (FACS). If the sorting is conducted prior to chromosomal analysis of the cells for the presence of artificial chromosomes, it provides a population of transfected cells that may be enriched for artificial chromosomes and thus facilitates any subsequent chromosomal analysis of the cells and identification and selection of cells containing an artificial chromosome, e.g., ACes. For example, the cells may be analyzed for indications of amplification of chromosomal segments, the presence of structures that may arise in connection with amplification and *de novo* artificial chromosome formation and/or the presence of artificial chromosomes, such as ACes. Analysis of the cells typically involves methods of visualizing chromosome structure, including, but not limited to, G- and C-banding and FISH analyses using techniques described herein and/or known to those of skill in the art. Such analyses can employ specific labelling of particular nucleic acids, such as satellite DNA sequences, heterochromatin, rDNA sequences and heterologous nucleic acid sequences, that may be subject to amplification. During analysis of transfected cells, a change in chromosome number and/or the appearance of distinctive, for example, by increased segmentation arising from amplification of repeat units, chromosomal structures will also assist in identification of cells containing artificial chromosomes.

**C. Purification of artificial chromosomes by Flow Cytometry and preparation of DNA from flow sorted chromosomes**

Artificial chromosomes were purified from the host cell by flow cytometry (see de Jong (1999) *Cytometry* 35:129-133). Briefly, purification was performed on FACS Vantage flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) equipped with a Trubo-Sort Option and two Innova 306 lasers (Coherent, Palo Alto, CA). The Turbo Sort Option modification include increasing the maximum system pressure from 20 lb/in<sup>2</sup> to 60 lb/in<sup>2</sup>, increasing the drop drive frequency from 50,000 drops/s to a maximum of 99,000 drops/s and increasing the deflection plate voltages from a maximum 6,000 V to 8,000 V.

Other modifications are made to the instrument to accommodate the higher pressures.

Hoechst 35258 was excited with the primary UV laser beam, and excitation detected in FL1 by using 420 nm hand-pass filter. Chromomycin A3 was excited by the second laser set at 458 nm and fluorescence detected in FL 4 by using a 475 nm long-pass filter. Both lasers had an output of 200 mW. Bivariate distributions (1,024 x 1024 channels) were accumulated during each sort. For all chromosome sorts, the sheath pressure was set at 30 lb/in<sup>2</sup> and a 50 µm diameter nozzle was installed. A drop delay profile was performed every morning and repeated after any major plug. Alignment of the instrument was performed daily by using 3.0 µm diameter Sphero rainbow beads (Spherotech, Libertyville, IL). Alignment was considered optimized when a CV of 2.0% or less was achieved for FL1 and FL4.

Condensing agents (hexylene glycol, spermine and spermidine) were added to the sheath buffer to maintain condensed chromosomes after sorting. The sheath buffer contains 15 nM Tris HCl, 0.1 mM EDTA, 20 mM NaCl, 1% hexylene glycol, 100 mM glycine, 20 µM spermine and 50 µM spermidine. The sorted chromosomes were collected in 1.5 ml screw-capped Eppendorf tubes at 4° C at a concentration of approximately 1 x 10<sup>6</sup> chromosomes/ml, which were then stored at 4° C.

For preparation of purified genomic DNA, sorted chromosome samples were brought to 0.5% SDS, 50 mM EDTA and 100 µg/ml Proteinase K, then incubated for 18 hours at 50°C. 1 µl of a 20 mg/ml glycogen solution (Boehringer Mannheim) was added to each sample, followed by extraction with an equal volume of Phenol: Chloroform: Isoamyl Alcohol (25:24:1). After centrifugation at 21,000Xg for 10 min, the aqueous phases were transferred to fresh microfuge tubes and were re-extracted as above. 0.2 volumes of 10 M NH<sub>4</sub>OAC, 1 µl of 20 mg/ml glycogen and 1 volume of iso-propanol were added to the twice extracted aqueous phases which were then vortexed and centrifuged for 15 minutes at 30,000Xg (at room temperature). Pellets were washed with 200 µl of 70% ethanol and re-centrifuged as above. The washed pellets were air-dried then resuspended in 5mM Tris-Cl, pH 8.0 at 0.5-2 X 10<sup>6</sup> chromosome equivalents/µl.

PCR was carried out on DNA prepared from sorted chromosome samples essentially as described (see, Co *et al.* (2000) *Chromosome Research* 8:183-191) using primers sets specific for EGFP and RAPSYN. Briefly, 50 µl PCR reactions were carried out on genomic DNA equivalent to 10,000 or 1000 chromosomes in a solution containing 10 mM Tris-Cl, pH 8.3, 50mM KCl, 200 µM dNTPs, 500 nM of forward and reverse primers, 1.5 mM MgCl<sub>2</sub>, 1.25 units Taq polymerase (Ampli-Taq, Perkin-Elmer Cetus, CA). Separate reactions were

carried out for each primer set. The reaction conditions were as follows: one cycle of 10 min. at 95°C, then 35 cycles of 1 min. at 94°C, 1 min. at 55°C, 1 min at 72°C, and finally one cycle of 10 min at 72°C. After completion the samples were held at 4°C until analyzed by agarose gel electrophoresis using the following primers (SEQ ID Nos. 1-4, respectively):

- 5 EGFP forward primer 5'-cgccaggaggcgaccatcttctt-3';  
EGFP reverse primer 3'-atcgcgcttcgtgggtcttt-3';  
RAPSYN forward primer 5'-aggactgggtggcttccaactcccagacac-3'; and  
RAPSYN reverse primer 5'-agcttctcattgctgcgcgcagggttcagg-3'.

All primers were obtained from Canadian Life Technologies, Burlington, ON.

10

## EXAMPLE 2

### Preparation of Cationic vesicles

Vesicles were prepared at a lipid concentration of 700 nmoles/ml lipid (cationic lipid/DOPE 1:1) as follows. In a glass tube (10ml) 350 nmol cationic lipid (SAINT-2) was mixed with 350 nmol dioleoyl-phosphatidylethanolamine (DOPE), both solubilized in an organic solvent (Chloroform, Methanol or Chloroform/Methanol 1:1, v/v).  
15 Dioleoylphosphatidylethanolamine (DOPE; Avanti Polar Lipids, Alabaster, AL) forms inverse hexagonal phases in a membrane and weakens the membrane. Other effectors that may be used are cis-unsaturated phosphatidylethanolamines, cis-unsaturated fatty acids and  
20 cholesterol. Cis-unsaturated phosphatidylcholines are less effective.

The solvent was evaporated under a stream of nitrogen (15 min/250 µl solvent at room temperature). The remaining solvent was removed totally by drying the lipid for 15 min in an desiccator under high vacuum from a vacuum pump. To the dried mixture was added 1 ml ultrapure water. This was vortexed vigorously for about 5 min. The resulting  
25 solution was sonicated in an ultrasonication bath (Laboratory Supplies Inc. NY) until a clear solution was obtained. The resulting suspension contained a population of unilamellar vesicles with a size distribution between 50 to 100 nm.

## EXAMPLE 3

### 30 Preparation of Cationic vesicles via alcoholic injection

In a glass tube (10ml) 350 nmol cationic lipid (Saint-2) was mixed with 350 nmol DOPE, both solubilized in an organic solvent (chloroform, methanol or chloroform/methanol 1/1). The solvent was evaporated under a stream of nitrogen (15 min/ 250 µl solvent at room

temperature). The remaining solvent was removed totally by drying the lipid for 15 min under high vacuum. This was then reconstituted in 100 µl pure ethanol.

#### EXAMPLE 4

##### 5 Transfection of beta ACes into V79-4 cell line

###### **Transfection procedure for various transfection agents**

All compounds were tested in a Chinese Hamster lung fibroblast line (V79-4, ATCC number CCL-39). Approximately 17 hours (2 cell doublings) prior to transfection, exponentially growing cells were trypsinized and plated at 250,000 cells per well into a 6 well petri dish with Dulbecco's Modified Eagle Medium (Life Technologies, Burlington, ON) and supplemented with 10% FBS (Can Sera, Rexdale ON)). At the time of transfection, the number of cells per well was estimated to be approximately 1 million. For transfection, each individual manufacturer's protocol for complexing to naked DNA was followed, with the exception that the amount of transfection agent used was varied, to reflect the different 10 amount and type of DNA present, as well as the different ionic strength of the complexing. One million ACes (in a volume of 800 µl) were typically combined with the transfection agent in a wide range of concentrations (between 5 times and 100 times the lowest manufacturers suggested concentration). The ACes/transfection mixture was allowed to 15 complex for the time recommended by the manufacturer, in volumes ranging from 0.8 ml to 20 1.9 ml; some manufacturers recommend adding media to the complexing reaction. The complexed mixture was then applied to the recipient cells and transfection allowed to proceed according to the manufacturer's protocol. Details on the various conditions used with different agents are presented in Table 1.

###### **Transfection procedure for Superfect agent**

25 Superfect was tested in a Chinese Hamster lung fibroblast line (V79-4, ATCC number CCL-39). Approximately 17 hours (2 cell doublings) prior to transfection, exponentially growing cells were trypsinized and plated at 250,000 cells per well into a 6 well petri dish with Dulbecco's Modified Eagle Medium (Life Technologies, Burlington, ON) and supplemented with 10% FBS (Can Sera, Rexdale ON)). One million ACes in 800µl of sort 30 buffer was complexed to 10µl of Superfect reagent. Complex was incubated at room temperature for 10 minutes. At the time of transfection, the number of cells per well was estimated to be approximately 1 million. Media was removed from wells and 600µl of DMEM and 10% FBS was added. Superfect:ACes complex was added to the wells drop-

wise and allowed to incubate for 3 hours at 37°C. After incubation, transfected cells were trypsinized and transferred to 15 cm dishes with 25 ml DMEM and 10% FBS and allowed to attach for 24 hours. After 24 hours, selection medium containing of 0.7 mg/ml hygromycin B was added to each well. The selection medium was changed every 2-3 days. After 10-12  
5 days colonies were screened for Beta-galactosidase expression and/or FISHed for detection of intact chromosome.

**Example of application of the determination of the Chromos Index**

Approximately 1 x 10<sup>6</sup> V79-4 cells were transfected with 1 x 10<sup>6</sup> IdUrd-labeled ACes complexed with a delivery agent (i.e., Lipofectamine PLUS and Lipofectamine or  
10 Superfect). The transfected cells were then fixed in ethanol. Fixed cells were denatured and exposed to FITC-conjugated antibody that specifically binds to BrdU/IdUrd-labeled nucleic acids.

The percentage of transfected cells containing IdUrd-labeled ACes was determined using flow cytometry and collecting FITC fluorescence. Data were accumulated to form  
15 bivariate channel distribution showing forward scatter versus green fluorescence (IdUrd-FITC). The fluorescence level at which cells were determined to be positive was established by visual inspection of the histogram of negative control cells such that the gate for the negative cells was set such that 1% appeared in the positive region.

The number of cells recovered at 24 hours post-transfection was determined by  
20 counting an aliquot using a Coulter Counter. To determine the control plating efficiency of a recipient cell line, the untreated cells were plated at 600-1000 cells per 10 cm petri dish in growth medium and left stationary in a 5% CO<sub>2</sub> incubator at 37°C for approximately five cell cycles or until average colony was made up of 50 cells. At this point the number of viable colonies was determined. The treated cells were seeded at 1000 cells if the CPE is above 0.1-  
25 0.2. If the CPE is low then the seeding density is increased to 5,000-50,000 cells per dish.

**EXAMPLE 5**

**Ultrasound mediated transfection of LMTK(-) cells with Lipofectamine**

LM(tk-) cells were grown at 37°C, 5% CO<sub>2</sub>, in DMEM with 4500 mg/L D-glucose,  
30 L-glutamine, pyridoxine hydrochloride and 10% Fetal Bovine Serum. The corner wells of a 12-well dish were seeded with 200,000 cells per well (this is to ensure no interference from the ultrasound waves from other wells) 24 hours before use.

The GFP chromosomes were counted to verify approximately 1X106 ACes per ml. The chromosomes were resuspended in the tube by flicking. Ten  $\mu$ l of chromosome suspension was removed and mixed with an equal volume of 30 mg/ml PI (propidium iodide) stain. Eight  $\mu$ l of the stained chromosomes was loaded onto a Petroff Hausser counting chamber and the chromosomes were counted.

5 The medium was removed from the cells, and the cells were washed twice with HBSS (without phenol red, Gibco BRL) warmed to 37°C. 500  $\mu$ l of the warmed HBSS was added to each well of cells (1  $\mu$ l) LipofectAMINE (Gibco BRL) was added to each well. The plates were then sealed with parafilm tape and shaken gently at 20 rpm at room temperature for 30 minutes (Stagger plates - 10 minutes for ease of handling).

10 After incubation Ultrasound gel (Other-Sonic Generic Ultra sound transmission gel, Pharmaceutical Innovations, Inc., Newark, NJ) was applied to the 2.5 cm sonoporation head. Ultrasound was applied with an ImaRX Sonoporation 100 at an output energy of 2.0 Watt/cm<sup>2</sup>, for 60 seconds, through the bottom of the plate of cells. After ultrasound of the 15 well one chromosome per seeded cell (2X105) or 200  $\mu$ l GFP ACes in sheath buffer (15 nM Tris HCl, 0.1 mM EDTA, 20 mM NaCl, 1% hexylene glycol, 100 mM glycine, 20  $\mu$ M spermine and 50  $\mu$ M spermidine) are added immediately to the well. (Repeat until all samples on the plate requiring ultrasound have been treated). The plate was then sealed once more with parafilm tape and shaken gently (20 rpm) for 1 hour at room temperature.

20 After the incubation 1 ml (DMEM with 4500 mg/L D-glucose, L-glutamine and pyridoxine hydrochloride, 10% Fetal Bovine Serum, and a 1x solution of penicillin and streptomycin from a 10000 units/ml penicillin and 10000 mg/ml Streptomycin, 100x stock solution) was added to each well and the cells were incubated 18-24 hours at 37°C.

25 The cells in the plates were then washed with antibiotic containing medium and 2 ml of medium was placed in each well. The cells continued to be incubated at 37°C with 5% CO<sub>2</sub> until 48 hours after transfection/sonoporation. The cells were then trypsinised and resuspended at a concentration of 1x 106 in DMEM to be analyzed by flow cytometry.

30 Results: Flow analysis was performed on a FACS Vantage (BDIS, San Jose, CA) equipped with a turbo-sort option and two Inova 305 lasers (Coherent, Palo Alto, CA). The GFP signal excitation is at 488 nm and the emission detected in FL1 using a 500nm long pass filter. Analysis of the transfected cells generated populations of GFP positive cells ranging from 13-27%. Non-sonoporated control value was 5%.

## EXAMPLE 6

### Ultrasound mediated transfection with Saint-2

#### A. Ultrasound mediated transfection of CHO-KI cells with Saint-2

CHO-KI cells were grown at 37°, 5% CO<sub>2</sub>, in CHO-S-SFM 2 Medium, (Gibco BRL,

5 Paisley, UK). Between 2 x 10<sup>5</sup> and 5 x 10<sup>5</sup> cells were plated onto sterile glass slides in a 12 well plate 24 h before usage.

Transfection of the cells was performed as follows. The medium was removed from the cells, and the cells were washed twice with HBSS (Hanks balanced salt solution without Phenol Red (Gibco BRL, UK)) at 37°C. Then 500µl HBSS at 37°C was added per well, 10 followed by 10 µl of the freshly prepared vesicle solution (prepared in Example 2) to yield a final concentration of 23.3 nmol/ml.

Alternatively, the medium was removed from the cells, and the cells were washed twice with HBSS. 500 µl HBSS/lipid solution at 37°C was added to each well. The HBSS/lipid solution was prepared by adding 1 µl ethanolic lipid solution (prepared as described above) to 500 µl HBSS under vigorous vortexing. The plates were then sealed with parafilm tape and shaken gently at room temperature for 30 min. After incubation, ultrasound was applied at an output energy of 0.5 Watt/cm<sup>2</sup> for 60 sec through the bottom of the plate to the cells. The ultrasound was mediated by an ultrasound gel (Aquasonic 100, Parker, NJ) between transducer and plate. The ultrasound was applied with an ImaRx Sonoporator 100. Immediately after applying ultrasound one GFP chromosome per seeded cell (2 x 10<sup>5</sup> - 5 x 10<sup>5</sup>) (prepared in Example 1) was added. The plate was then sealed again and shaken gently for 1 h at room temperature. After the incubation 1ml medium (CHO-S-SFM 2 with 10% Fetal Calf Serum, 10000 µg/ml Penicillin and 10000 µg/ml Streptomycin Gibco BRL, Paisley, UK) was added to each well and the cells were incubated for 24 h at 25 37°C. The cells were then washed with medium and 1 ml medium was added and the cells were incubated at 37° for another 24 h. Detection of expressed genes was then assayed by microscopy or detection of the transferred chromosome by FISH analysis. The negative control was performed in the same way, but with no chromosomes added to the cells.

#### Results

30 After transfection, using visual inspection, 30% of the cells remained on the glass slide of which 10% were positive for green fluorescent protein expression after 48 hours (3% of original population). After culturing for two weeks, FISH was performed on the cells and 1.4% of the cells contained an intact artificial chromosome.

**B. Ultrasound mediated transfection of Hep-G2 cells with Saint-2**

Hep-G2 cells were grown at 37°C, 5% CO<sub>2</sub>, in DMEM with 4500 mg/l Glucose, with Pyridoxine/HCL, 10% Fetal Calf Serum, 10000 µg/ml Streptomycin and 1000 µg/ml Penicillin. Between 2 x 10<sup>5</sup> and 5 x 10<sup>5</sup> cells were plated onto sterile glass slides in a 12 wells plate 24 hours before usage.

Cells were transfected with GFP chromosomes using the procedure of Example 6A except that the CHO-KI medium was replaced with Hep-G2 medium.

**Results**

After transfection, 30% of the cells remained on the glass slide. 80% of these cells 10 were positive for green fluorescent protein expression.

**C. Ultrasound mediated transfection of A9 cells with Saint-2**

A9 cells were grown at 37°C, 5% CO<sub>2</sub>, in DMEM with 4500 mg/l Glucose, with Pyridoxine/HCL, 10% Fetal Calf Serum, 10000 µg/ml Streptomycin and 10000 µg/ml Penicillin (GIBCO BRL, Paisley, UK). Between 2 x 10<sup>5</sup> and 5 x 10<sup>5</sup> cells were plated onto 15 sterile glass slides in a 12 well plate 24 h before usage.

Cells were transfected with GFP chromosomes using the procedure of Example 6A except that CHO-KI medium was replaced with A9 medium.

**Results**

After transfection, 30% of the cells remained on the glass of which 50% were positive 20 for green fluorescent protein expression.

**EXAMPLE 7****Delivery of ACes into synoviocytes, skeletal muscle fibroblasts and skin fibroblasts**

A mammalian (murine) ACes artificial chromosome (~60 Mb) containing primarily 25 murine pericentric heterochromatin, and including a reporter gene (*lacZ*) and a hygromycin B selectable marker gene, prepared as described in U.S. Patent Nos. 6,025,155 and 6,077,697 and PCT Application Publication No. WO 97/40183 was delivered into primary rat 30 fibroblast-like synoviocytes, rat skin fibroblasts and a rat skeletal muscle fibroblast cell line (L8 cells; ATCC Accession No. CRL-1769). Prior to delivery, the ACes were labelled with iododeoxyuridine (IdUrd) as described herein.

**Preparation of cells**

Primary fibroblast-like synoviocytes and rat skin fibroblasts were obtained from rats using standard methods [see, e.g., Aupperle *et al.* (1999) *J. Immunol.* 163:427-433 and

Alvaro-Garcia *et al.* (1990) *J. Clin. Invest.* 86:1790]. Such methods include isolation of synoviocytes from rodent knees generally by removal of skin and muscle, followed by mincing of knee joint tissue. The minced tissue is then incubated with collagenase, filtered through nylon mesh and washed extensively. Cells can be cultured overnight, after which time non-adherent cells are removed. Adherent cells can be cultured and passaged by replating at a dilution when the cultures reach confluence. The cells were plated at 50,000-75,000 cells per 6-well dish in media containing low glucose DMEM, l-glutamine, penicillin/streptomycin and 20% FBS. The cells were grown in a 5% CO<sub>2</sub> incubator at 37°C for 3-5 days until approximately 80% confluence or 500,000 cells per well.

10           **Transfection of cells with ACes**

One million IdUrd-labelled ACes were complexed with 2, 5 or 10 µl of Superfect (Qiagen) or Lipofectamine Plus (Life Technologies; Gibco) as follows. Complexing with Superfect was conducted for 10 minutes at room temperature. For complexing with Lipofectamine Plus, the indicated amounts of PLUS reagent were added to 1 million ACes and complexed at room temperature for 15 minutes. Next the indicated amounts of Lipofectamine were added into 200 µl of low glucose DMEM (no FBS) and combined with the ACes/PLUS complex for 15 minutes at room temperature. The complexed ACes were then added dropwise to the cells in 600 µl media (final volume of approximately 1.4 ml). After 3 hrs at 37°C in a 5% CO<sub>2</sub> incubator, a total volume of 3 ml of culture media (low glucose DMEM, l-glutamine, penicillin/streptomycin and 20% FBS) was added. After 24-48 hrs, the cells were trypsinized to form a single cell suspension, centrifuged to remove the supernatant and then fixed in cold 70% ethanol for a minimum of one hour. An aliquot of the fixed cells was saved for microscopic analysis.

25           **FITC-Conjugated antibody labelling of ACes**

Following transfection, the ACes were labelled with FITC-conjugated antibody that specifically binds to BrdU- or IdUrd-labelled nucleic acids and the cells were analyzed by FACs for FITC fluorescence and microscopic staining. Fixed cells were denatured in 2N HCl and 0.5% Triton-X for 30 minutes at room temperature. After denaturation, the cells were neutralized by a series of wash steps at 4°C. To minimize background staining, the sample was resuspended in PBS and 4% FBS or BSA and 0.1% Triton-X (blocking buffer) for a minimum of 15 minutes. The cells were then pelleted and exposed for 2 hours at room temperature to FITC-conjugated antibody. After the cells were washed with blocking buffer, the sample was ready for flow cytometry analysis. Samples for microscopic analysis were

dried on slides and the above staining protocol was followed, except that BrdU/IdUrd antibody was diluted 1/5 and exposed to cells for 24 hours.

### Results

The delivery of intact ACes was detected within 24 to 48 hours post transfection. The 5 number of cells recovered at 24 hours post-transfection was determined by counting an aliquot using a Coulter Counter. To determine the control plating efficiency of a recipient cell line or plating efficiency of the transfected cells, the cells were plated at 1000-10,000 cells per 10 cm petri dish in growth medium and left stationary in a 5% CO<sub>2</sub> incubator at 37°C for 10 days. At that point, the number of viable colonies was determined. The 10 normalized plating efficiency was calculated as described herein.

When Superfect was used as a delivery agent, the percent delivery into fibroblast-like synoviocytes as determined by flow cytometry ranged from ~24% to ~66.3%. The normalized % plating efficiency was ~36% when 2 µl of Superfect was used and ~16% when 5 µl of Superfect was used. Higher doses of Superfect were associated with toxicity and 15 multiple ACes per cell as compared to lower doses. When Lipofectamine Plus was used as a delivery agent, the percent delivery into fibroblast-like synoviocytes as determined by flow cytometry ranged from ~11% to ~27% with percent delivery increasing with increasing doses of agent.

L8 and rat skin fibroblasts (RSF) that had been transfected with ACes were grown 20 under hygromycin B selection and analyzed for lacZ expression. While in this example, a hygromycin selection gene was included in the ACes, there are numerous other selectable marker genes that may be used in connection with the transfer of heterologous nucleic acids into cells when it is desirable to include such genes. Such selection systems are known to those of skill in the art. A choice of selectable marker gene can, for instance, take into 25 account the level of toxicity of the selection agent on the host cell for transfection. Identification of an appropriate selectable marker gene is routine employing the guidance provided herein.

Clones of L8 cells and RSF cells expressing lacZ were identified. These results demonstrate that IdUrd-labelled ACes can be delivered efficiently into primary cells as well 30 as cell lines and that transgenes contained in the ACes are expressed in the transfected cells.

## EXAMPLE 8

### *Ex vivo transfer of reporter genes into rat joints*

To examine transfer of a heterologous gene into an *in vivo* environment and expression of the gene *in vivo*, L8 cells transfected with ACes as described above were injected into the ankle joint of rats with adjuvant-induced arthritis. On day 0, adjuvant induction of arthritis was performed on Lewis rats. Methods for adjuvant induction of arthritis in animal models are known in the art [see, e.g., Kong *et al.* (1999) *Nature* 4023:304-309]. In one exemplary protocol for adjuvant induction of arthritis, Lewis rats are immunized at the base of the tail with 1 mg Mycobacterium tuberculosis H37 RA (Difco, Detroit, Michigan) in 0.1 ml mineral oil on day 0. Paw swelling typically begins around day 10.

On day 12, intra-articular injection of transfected L8 cells (~0.7 x 10<sup>6</sup> cells) or untransfected control cells into the right ankle joint was performed. On day 14, the rats were sacrificed in order to analyze the joints for the presence of transplanted transfected L8 cells.

Different tissues of the sacrificed rats were examined by RT-PCR analysis for the presence of lacZ mRNA. Total mRNA was extracted from the tissues and RT-PCR was performed using primers specific for the lacZ gene. The an amplification product was detected only in the synovium, and not in the other tissues (liver, kidney, heart, spleen and lung). Synovium from the sacrificed rats was also analyzed by *in situ* enzymatic staining X-gal staining for β-gal activity. After snap freezing of the synovium, 8 μm and 20 μm sections were cut, counter-stained with Mayer's hematoxylin, and analyzed for blue staining of the cells. Staining was detected in synovium injected with L8 cells that had been transfected with ACes but not in synovium injected with untransfected cells. These results demonstrate successful *ex vivo* gene transfer in a rat adjuvant arthritis model using ACes containing a marker gene and thus the feasibility of treating arthritis and other connective tissue diseases using ACes as non-viral vectors for gene therapy.

## EXAMPLE 9

### *A flow cytometry technique for measuring delivery of artificial chromosomes*

Production cells lines (see Example 1) were grown in MEM medium (Gibco BRL) with 10% fetal calf serum (Can Sera, Rexdale ON) with 0.168 μg/ml hygromycin B (Calbiochem, San Diego, CA). Iododeoxyuridine or Bromodeoxyuridine was added directly to culture medium of the production cell line (CHO E42019) in the exponential phase of growth. Stock Iododeoxyuridine was made in tris base pH 10. Bromodeoxyuridine stocks in

PBS. Final concentrations of 0.05-1  $\mu$ M for continuous label of 20-24 hours of 5-50  $\mu$ M with 15 minute pulse. After 24 hours, exponentially growing cells were blocked in mitosis with colchicine (1.0  $\mu$ g/ml for 7 hours before harvest. Chromosomes were then isolated and stained with Hoechst 33258 (2.5  $\mu$ g/ml) and chromomycin A3 (50  $\mu$ g/ml). Purification of 5 artificial chromosomes was performed using a FACS Vantage flow cytometer (Becton Dickinson Immunocytometry systems, San Jose, CA). Chromomycin A3 was excited with the primary laser set at 457 nm, with emission detected using 475 nm long pass filter. Hoechst was excited by the secondary UV laser and emission detected using a 420/44 nm band-pass filter. Both lasers had an output of 150 mW. Bivariate distribution showing cell karyotype 10 was accumulated from each sort. ACes were gated from other chromosomes and sorted. Condensing agents (hexylene glycol, spermine, and spermidine) were added to the sheath buffer to maintain condensed intact chromosome after sorting. IdU labeling index of sorted chromosomes was determined microscopically. Aliquot (2-10  $\mu$ l) of sorted chromosomes 15 was fixed in 0.2% formaldehyde solution for 5 minutes before being dried on clean microscopic slide. Microscope sample was fixed with 70% ethanol. Air-dried slide was denatured in coplin jar with 2N HCl for 30 minutes at room temperature and washed 2-3 times with PBS. Non specific binding was blocked with PBS and 4% BSA or serum for minimum of 10 minutes. A 1/5 dilution of FITC conjugated IdU/BrdU antibody (Becton Dickinson) with a final volume of 60-100  $\mu$ l was applied to slide. Plastic strips, Durra seal 20 (Diversified Biotech, Boston, MA) were overlaid on slides, and slides were kept in dark at 4% C in humidified covered box for 8-24 hours. DAPI (Sigma) 1  $\mu$ g/ml in Vectorshield was used as counterstain. Fluorescence was detected using Zeiss axioplan 2 microscope equipped for epiflorescence. Minimum of 100 chromosomes was scored for determining % labeled. Unlabeled chromosomes were used as negative control.

25 The day before the transfection, trypsinize V79-4 (Chinese Hamster Lung fibroblast) cells and plate at 250,000 into a 6 well petri dish in 4 mls DMEM (Dulbecco 's Modified Eagle Medium, Life Technologies) and 10% FBS (Can Sera Rexdale ON). The protocol was modified for use with LM (tk-) cell line by plating 500,000 cells. Lipid or dendrimer reagentwas added to 1 X106 ACes sorted in ~800  $\mu$ l sort buffer. Exemplary protocol 30 variations are set forth in Table 1. Chromosome and transfection agents were mixed gently. Complexes added to cells drop-wise and plate swirled to mix. Plates were kept at 37°C in a 5% CO<sub>2</sub> incubator for specified transfection time. The volume in a well was then made up to 4-5 ml with DMEM and 10% FBS. Recipient cells left for 24 hours at 37°C in a 5% CO<sub>2</sub>

incubator. Trypsinize transfected cells. Samples to be analyzed for IdU labeled chromosome delivery are fixed in cold 70% ethanol and stored at -20°C, to be ready for IdU antibody staining. Samples to be grown for colony selection are counted and then transferred to 10-cm dishes at densities of 10,000 and 100,000 cells in duplicate with remaining cells put in a 15 cm dish. After 24 hours, selection medium containing of DMEM and 10% FBS with 0.7 mg/ml hygromycin B, # 400051 (Calbiochem San Diego, CA) is added. Selection medium is changed every 2-3 days. This concentration of hygromycin B kills the wild type cells after selection for 7 days. At 10-14 days colonies were expanded and then screened by FISH for intact chromosome transfer and assayed for beta galactosidase expression.

10

**Table 1: Delivery Transfection Protocols**

Agent	Dilution Stock	Pre treatment of ACes	Complexing time (minutes)	Added to complexes	Medium (ml) added to wells before complexes	Transfection time (hours)
CLONFECTIN	2-8 µg in NaCl-HEPES buffer		20	1.8 ml of serum free medium		4
CYTOFECTENE			10-20	200 µl of 50% FBS plus DMEM		24
ENHANCER + EFFECTENE (1:5 ratio)		Enhancer 5 minutes	10		1.2	3
EU-FECTIN-1 to 11			5-10			6
FUGENE 6	0.5-6 µl to final volume of 100 µl in serum free medium		15-45			4
GENEPORTER 2	2.5 µl added to 150 µl of serum free medium		2-10			2-4
LIPOFECTAMIN E			15			3
LIPOFECTAMIN E 2000			20		2.5	5
METAFFECTENE	diluted into 60 µl serum free medium		15-45		0.8	6
PLUS + LIPOFECTAMIN E (1:1 and 3:2 ratio)		PLUS and 200 µl of DMEM for 15 minutes	15			3
SUPERFECT			10		0.6	3

## IDU ANTIBODY LABELING

A standard BrdU staining flow cytometry protocol (Gratzer *et al.* Cytometry (1981);6:385-393) was used except with some modifications at the neutralization step, the presence of detergent during denaturation and the composition of blocking buffer. Between each step samples are centrifuged at 300 g for 7-10 minutes and supernatant removed. Samples of 1-2 million cells are fixed in 70% cold ethanol. Cells are then denatured in 1-2 ml of 2N HCL plus 0.5% triton X for 30 minutes at room temperature. Sample undergoes 3-4 washes with cold DMEM until indicator is neutral. Final wash with cold DMEM plus 5% FBS. Blocking/permeabilization buffer containing PBS, 0.1% triton X and 4%. FBS is added for 10-15 minutes before pelleting sample by centrifugation. Add 20 µl of IdU/BrdU FITC conjugated B44 clone antibody (Becton Dickinson Immunocytometry Systems, San Jose, CA) to pellet and leave for 2 hours at room temperature in the dark with agitation every 30 minutes. Wash cells with block/permeabilization buffer and resuspend in PBS for flow analysis.

## FLOW CYTOMETRY DETECTION OF FLUORESCENT IDUrD LABELED ACES

### ACes

Percentage of transfected cells containing IdU labeled ACes was determined using a flow cytometry with an argon laser turned to 488 nm at 400 mW. FITC fluorescence was collected through a standard FITC 530/30-nm band pass filter. Cell populations were gated on the basis of side scatter versus forward scatter to exclude debris and doublets. Data was accumulated (15,000 events) to form bivariate channel distribution showing forward scatter versus green fluorescence (IdU-FITC). The fluorescence level at which cells were determined to be positive was established by visual inspection of the histogram of negative control cells, such that approximately 1% appeared in the positive region.

### Results:

The transfection delivery results of IdU labeled ACes are set forth in Table 2.

**Table 2**

COMPOUND	DOSE Microliters agent added per 1	DELIVERY	
		% IdU positive (24 hours)	
CLONFECTIN	6	0.61	
CYTOFECTENE	8	14.67	
ENHANCER + EFFECTENE (1:5)	1.6,10	17.08	
EU-FECTIN-1	10	4.57	
EU-FECTIN-2	5	0.14	

EU-FECTIN-3	10	0.69
EU-FECTIN-4	10	0.24
EU-FECTIN-5	10	0.41
EU-FECTIN-6	10	0.46
EU-FECTIN-7	10	1.21
EU-FECTIN-8	10	1.58
EU-FECTIN-9	10	0.6
EU-FECTIN-10	10	0.77
EU-FECTIN-11	5	1
FUGENE	8	0.49
GENEPORTER	5	22.12
LIPOFECTAMINE	26	17.81
LIPOFECTAMINE 2000	30	10.96
PLUS + LIPOFECTAMINE (1:1)	12, 12	12.2
PLUS + LIPOFECTAMINE (3:2)	24,16	26.97
METAFFECTENE	10	14.14
SUPERFECT	2	27.67

### EXAMPLE 10

#### Delivery of ACes into LM(tk-) and Hff cells by cell synchronization

A mammalian (murine) derived 60Mb ACes artificial chromosome was used for all transfections. The ACes artificial chromosome consists primarily of mouse major satellite DNA, interspersed with blocks of ‘payload’ genetic material, including a reporter gene (*lacZ*) and a hygromycin B selectable marker gene. The ACes were prepared and purified as previously described herein and delivered into synchronized, immortalized murine LM(tk-) and primary human foreskin fibroblast (Hff) cells. Two common cell cycle arrest agents, thymidine and nocodazole, were chosen for cell synchronization. Thymidine incorporation accumulates cells in G0-G1 phase, and nocodazole acts by arresting cells in G2-M. Removal of the arresting agents allows the cells to continue through the cell cycle in a synchronous manner for approximately 24 hours. Two cationic transfection agents were used to deliver the ACE systems into cells, Superfect (Qiagen, Inc., Mississauga, Ontario) and 10 LipofectAMINE PLUS (Gibco BRL). Transfection with these agents was carried out according to manufacturer’s protocols.

#### Nocodazole Cell Synchronization

##### Optimization of Nocodazole Cell Synchronization in LM(tk-) Cells

To determine the optimum blocking concentration of nocodazole, nocodazole concentrations were titrated against LM(tk-) cells to generate a dose response. Timing of the optimal blocking concentration (100 ng/ml) was then assessed over 0-16 hours using flow cytometry. To determine the cell cycle kinetics of LM(tk-) cell populations at different time points, cells were stained with propidium iodide (PI) at a concentration of 20 µg/ml for 1x10<sup>6</sup>

cells and analyzed with a FACS Vantage SE (Becton Dickinson Immunocytometry Systems, San Jose, CA) equipped with an argon laser tuned to 488 nm at 200 mW. Whole intact cells were gated on forward scatter (FSC, representing cell size) versus side scatter (SSC, representing internal granularity) and PI emission was detected with a 630/22 filter. A list mode file was generated with a minimum of 20,000 whole intact cells analyzed. The proportion of cells in G0-G1, S and G2-M were documented for each time point. Optimum cell-cycle arrest at the G2/M boundary was achieved by incubating cells with 100 ng/ml nocodazole in serum containing DMEM medium for 6 hours. After 6 hours of nocodazole exposure at 100 ng/ml, approximately 20% of cells were in S phase and 50% of cells were at G2/M. Cell cycle kinetics were again monitored by flow cytometry upon release of the nocodazole block and at frequent intervals over a 16 hour period.

#### Optimizing the Time Point for Transfection in LM(tk-) cells

Cells were plated at 7.5x10<sup>5</sup> cells per well of a 6-well plate 24 hours prior to nocodazole treatment. The cells were incubated with nocodazole for 6 hours. After the 6 hour incubation, wells were hosed with medium to dislodge the suspension fraction of cells. Nocodazole containing medium was removed and the wells were washed with fresh medium. The wash medium was aspirated and fresh medium was replaced in the wells. To determine the optimum time point for transfection, different cell samples were transfected at different time points after release of the nocodazole block. The time points were 0, 2, 4, 6, 8, 12, and 16 hours after release of the nocodazole block. Following transfer, the cells were plated and transfecants identified using hygromycin selection. Highest colony formations were achieved when transfection occurred 2-4 hours post nocodazole block. At these time points, cell population distribution consisted of approximately 10% of cells in G0/G1, 20% in S, and 70% in G2/M. Transfectants were analyzed using FISH to determine the quality of the transferred material. Randomly selected drug resistant colonies were analyzed and the number of colonies where at least 50% of the cells had an intact ACes were scored. Selected colonies derived from nocodazole synchronized transfections had a larger percentage of cells that maintained intact ACes compared with asynchronous control colonies. With both LipofectAMINE PLUS or Superfect, the percentage of colonies meeting this criteria was 4% (2/49) for asynchronous control colonies and 43% (20/47) for nocodazole synchronized colonies.  $\beta$ -galactosidase staining was used to confirm expression of the reporter gene.

**Delivery of ACes into LM(tk-) cells by cell synchronization.**

Cells were synchronized by nocodazole treatment (100ng/ml final concentration) for 6 hours prior to transfection. The mitotic cells are gently removed by rinsing the plate with growth medium. Adherent cells were transfected at 2-hour intervals (approximately 1 million 5 cells per interval) with Superfect (10ul) over an 8-hour time course. Twenty-four hours post-transfection the cells are counted (Coulter counter) and a small fraction are plated for a modified toxicity assay. The remaining cells were plated separately in growth medium and 24 hours later were placed in selection medium (growth medium with 1mg/ml hygromycin B). The selection medium was changed every 2-3 days until colonies formed.  
10 LipofectAMINE PLUS concentrations used were 12ul for both the LipofectAMINE and PLUS reagent. Transfection conditions were otherwise identical and selection of LM(tk-) cells was accomplished as described above.

**Delivery of ACes into Hff cells by cell synchronization.**

The protocol for Hff cells differs from that used for LM(tk-) cells in the following 15 details: nocodazole concentration was 45ng/ml for an 8 hour exposure; 4ul Superfect was used; Transfection were carried out an half hour intervals over a two hour time frame; colony selection was not done as the Hff cells are hygromycin B sensitive. Cells were transfected with either 4ul Superfect or 10ul LipofectAMINE PLUS (10ul for each component 20 LipofectAMINE and PLUS; Invitrogen). Transfections were carried out every half hour over a 2-hour timeframe (cells returned to logarithmically growing state after this short time). The Hff cells are sensitive to hygromycin B therefore transfections were carried out using the RFP ACes and expression was monitored by flow cytometry.

**Thymidine Cell Synchronization****Optimization of Thymidine Cell Synchronization in LM(tk-) Cells**

To determine the optimum parameters for cell cycle synchronization, thymidine was 25 administered to cell samples in a dose range of 1-20 mM. Samples were analyzed by flow cytometry to determine the cell cycle kinetics of LM(tk-) cell populations at frequent intervals over a 16 hour time period. Optimum synchronization of cells in G0/G1 was achieved by incubating LM(tk-) cells with 10 mM thymidine in serum containing DMEM 30 medium for 16 hours. The thymidine block was released by washing the cells twice in serum-free medium and replacing the medium with complete medium containing 24 µM 2'- deoxycytidine 5 'mono-phosphate (dCMP; Sigma D-7750, St. Louis, MO). Cell cycle

kinetics were again monitored by flow cytometry upon release of the thymidine block and at frequent intervals over a 16 hour period.

#### Optimizing the Time Point for Transfection in LM(tk-) Cells

Cells were plated at 7.5x10<sup>5</sup> cells per well of a 6-well plate 24 hours prior to

5 thymidine treatment. The cells were incubated with medium containing 10 mM thymidine for 16 hours. After the 16 hour incubation, the thymidine block was released by washing the cells twice in serum-free medium and replacing the medium with 24 µM deoxycytidine 5 'mono-phosphate (dCMP) supplemented complete medium. To determine the optimum time point for transfection, different cell samples were transfected at different time points 10 after release of the thymidine block. The time points were 0, 2, 4, and 6 hours after release of the thymidine block. Following transfer, the cells were plated and transfectants identified using hygromycin selection. Transfectants were analyzed using FISH to determine ACE system integrity and β- galactosidase staining to confirm expression of the reporter gene. Optimal transfection was achieved at 4-5 hours post thymidine release.

15 **Hff cells**

A protocol similar to that used with the LM(tk-) cells was employed with the exception of an alteration in the dose of thymidine (5mM).

#### Results

Studies indicate successful achievement of reversible arrest of the LM(tk-) and Hff 20 cells with both nocodazole and thymidine. Studies further show that synchronization and release of cells at specific points in the cell cycle increases the efficiency of ACes transfection into LM(tk-) and Hff cells. Using nocodazole or thymidine with either of two 25 transfection agents (Superfect or LipofectAMINE PLUS), transfection efficiencies were enhanced 2.5-6 fold above control transfections on asynchronous cell populations. Transfection efficiency was strongly correlated with the fraction of cells in G2/M ( $R^2 = 30$  0.827), regardless of which blocking agent was used suggesting that optimal transfection is achieved when the majority of the cells are in the G2/M phase of the cell cycle, regardless of where the cell population is originally arrested. Qualitative assessments of the chromosomes also revealed that delivery of ACes to cells during the optimized transfection point led to higher proportions of intact artificial chromosomes compared to asynchronous controls.

Since modifications will be apparent to those of skill in this art, it is intended that this invention be limited only by the scope of the appended claims.

**CLAIMS:**

1. A method for introducing a large nucleic acid molecule into a cell, comprising:
  - obtaining a cell that is in a pre-selected phase; and
  - contacting the cell with the large nucleic acid molecule, whereby the large nucleic acid molecule is delivered into the cell.
2. The method of claim 1, wherein the nucleic acid molecule is greater than about 0.6 megabase.
3. The method of claim 1, wherein the nucleic acid molecule is greater than about 1 megabase.
4. The method of claim 1, wherein the nucleic acid molecule is greater than about 5 megabases.
5. The method of claim 1, wherein the nucleic acid molecule is a natural chromosome, an artificial chromosome, or a fragment of a chromosome that is greater than about 0.6 megabase or naked DNA that is greater than about 0.6 megabase.
6. The method of claim 1, wherein the nucleic acid molecule is an artificial chromosome.
7. The method of claim 1, wherein the nucleic acid molecule is an artificial chromosome expression system (ACes).
8. The method of claim 1, wherein the nucleic acid molecule and/or the cell is exposed to a delivery agent.
9. The method of claim 8, wherein the delivery agent comprises a cationic compound.
10. The method of claim 9, wherein the cationic compound is selected from the group consisting of a cationic lipid, a cationic polymer, a mixture of cationic lipids, a mixture of cationic polymers, a mixture of a cationic lipid and a cationic polymer, a mixture of a cationic lipid and a neutral lipid, polycationic lipids, non-liposomal forming lipids, activated dendrimers, and a pyridinium chloride surfactant.
11. The method of claim 8, wherein the delivery agent is a composition that comprises one or more cationic compounds, wherein the compound is selected from the group consisting of 2,3-dioleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate (DOSPA), C<sub>52</sub>H<sub>106</sub>N<sub>6</sub>O<sub>4</sub>.4CF<sub>3</sub>CO<sub>2</sub>H, C<sub>88</sub>H<sub>178</sub>N<sub>8</sub>O<sub>4</sub>S<sub>2</sub>.4CF<sub>3</sub>CO<sub>2</sub>H, C<sub>40</sub>H<sub>84</sub>NO<sub>3</sub>P.CF<sub>3</sub>CO<sub>2</sub>H, C<sub>50</sub>H<sub>103</sub>N<sub>7</sub>O<sub>3</sub>.4CF<sub>3</sub>CO<sub>2</sub>H, C<sub>55</sub>H<sub>116</sub>N<sub>8</sub>O<sub>2</sub>.6CF<sub>3</sub>CO<sub>2</sub>H, C<sub>49</sub>H<sub>102</sub>N<sub>6</sub>O<sub>3</sub>.4CF<sub>3</sub>CO<sub>2</sub>H, C<sub>44</sub>H<sub>89</sub>N<sub>5</sub>O<sub>3</sub>.2CF<sub>3</sub>CO<sub>2</sub>H, C<sub>100</sub>H<sub>206</sub>N<sub>12</sub>O<sub>4</sub>S<sub>2</sub>.8CF<sub>3</sub>CO<sub>2</sub>H, C<sub>41</sub>H<sub>78</sub>NO<sub>8</sub>P) C<sub>162</sub>H<sub>330</sub>N<sub>22</sub>O<sub>9</sub>.13CF<sub>3</sub>CO<sub>2</sub>H,

$C_{43}H_{88}N_4O_2.2CF_3CO_2H$ ,  $C_{43}H_{88}N_4O_3.2CF_3CO_2H$ , and (1-methyl-4-(1-octadec-9-enyl-nonadec-10-enylenyl) pyridinium chloride.

12. The method of claim 1, wherein:

the nucleic acid molecule is exposed to an agent that increases contact between the

5 nucleic acid molecule and the cell; and

the cell is exposed to an agent that enhances permeability of the cell.

13. The method of claim 12, wherein the exposure of the cell to an agent that enhances permeability comprises applying ultrasound or electrical energy to the cell.

14. The method of claim 1, wherein the cell is selected from the group consisting of a nuclear transfer donor cell, a stem cell, a primary cell, a cell from an immortalized cell line, an embryonic cell, a tumor cell, a transformed cell and a cell capable of the generation of a specific organ.

15. The method of claim 1, wherein the cell is selected from the group consisting of a primary cell, an immortalized cell, an embryonic cell, a stem cell, a transformed cell and a tumor cell.

16. The method of claim 1, wherein the cell is selected from the group consisting of a nuclear transfer donor cell, a stem cell, and a cell capable of the generation of a specific organ.

17. The method of claim 1, wherein the cell is a mammalian cell.

18. The method of claim 1, wherein the cell is a rodent cell or a human cell.

20 19. The method of claim 1, wherein the cell is a fibroblast.

20. The method of claim 1, wherein the cell is a synoviocyte.

21. The method of claim 20, wherein the cell is a fibroblast-like synoviocyte.

22. The method of claim 1, wherein the pre-selected phase of the cell is determined by a method comprising:

25 introducing a large nucleic acid into cells at different phases;  
determining and comparing the efficiency of delivery and/or of transfection of the nucleic acid into the cells at different phases; and

selecting a phase at which the efficiency of delivery and/or of transfection is increased relative to efficiency of delivery and/or transfection at other phases.

30 23. The method of claim 1, wherein the cell that is in a pre-selected phase is obtained by a method comprising:

exposing one or more cells to a cell cycle arrest agent;

exposing the one or more cells to conditions that permit cell cycling;

selecting a cell that is in the pre-selected phase.

24. The method of claim 23, wherein the arrest agent is an anti-microtubule agent.
25. The method of claim 23, wherein the arrest agent is nocodazole or thymidine.
26. The method of claim 1 or 23, wherein the pre-selected phase is G2/M.
- 5 27. The method of claim 23, wherein exposure of the one or more cells to an arrest agent results in arrest of the one or more cells in mitosis or G0/G1.
28. The method of claim 1, wherein the nuclear membrane of the cell is absent.
29. The method of claim 1, wherein a plurality of cells in a pre-selected phase is obtained.
30. The method of claim 29, wherein the plurality of cells is in a synchronous population
- 10 of cells.
  31. The method of claim 30, wherein the efficiency of transfection of the large nucleic acid molecule to cells in a synchronous population of cells is at least 1.5, at least 2, at least 2.5, at least 3, at least 3.5, at least 4, at least 4.5, at least 5, at least 5.5, at least 6, at least 6.5, at least 7, at least 7.5, at least 8, at least 8.5, at least 9, at least 9.5 or at least 10-fold greater than the efficiency of transfection of the large nucleic acid molecule to cells in an asynchronous population of cells.
  - 15 32. The method of claim 30, wherein the large nucleic acid molecule is a chromosome or artificial chromosome and the percentage of cells comprising an intact delivered chromosome after contact of a synchronous population of cells with the chromosome or artificial chromosome is greater than the percentage of cells comprising an intact delivered chromosome after contact of an asynchronous population of cells with the chromosome or artificial chromosome.
  - 20 33. The method of claim 32 wherein the artificial chromosome is an ACes.
  34. A method for introducing a chromosome or functional fragment thereof into a cell, comprising contacting the cell with a chromosome or fragment thereof that is in the same or similar phase as the cell, whereby the chromosome or functional fragment thereof is delivered into the cell.
  - 25 35. The method of claim 34 wherein the chromosome is an artificial chromosome.
  36. The method of claim 35, wherein the chromosome is an ACes.
  - 30 37. A method for *ex vivo* gene therapy, comprising:
    - obtaining a cell that is in a pre-selected phase;
    - contacting the cell with a nucleic acid molecule, whereby the nucleic acid molecule is delivered into the cell; and

introducing the cell into a subject.

38. The method of claim 37, wherein the nucleic acid molecule is a large nucleic acid molecule.

39. The method of claim 37, wherein the nucleic acid molecule is greater than about 0.6 megabase.

40. The method of claim 37, wherein the nucleic acid molecule is greater than about 1 megabase.

41. The method of claim 37, wherein the nucleic acid molecule is greater than about 5 megabases.

10 42. The method of claim 37, wherein the nucleic acid molecule is a natural chromosome, an artificial chromosome, or a fragment of a chromosome that is greater than about 0.6 megabase or naked DNA that is greater than about 0.6 megabase.

43. The method of claim 37, wherein the nucleic acid molecule is an artificial chromosome.

15 44. The method of claim 37, wherein the nucleic acid molecule is an artificial chromosome expression system (ACes).

45. The method of claim 37, wherein the nucleic acid molecule and/or the cell is exposed to a delivery agent.

46. The method of claim 45, wherein the delivery agent comprises a cationic compound.

20 47. The method of claim 46, wherein the cationic compound is selected from the group consisting of a cationic lipid, a cationic polymer, a mixture of cationic lipids, a mixture of cationic polymers, a mixture of a cationic lipid and a cationic polymer, a mixture of a cationic lipid and a neutral lipid, polycationic lipids, non-liposomal forming lipids, activated dendrimers, and a pyridinium chloride surfactant.

25 48. The method of claim 45, wherein the delivery agent is a composition that comprises one or more cationic compounds, wherein the compound is selected from the group consisting of 2,3-dioleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate (DOSPA), C<sub>52</sub>H<sub>106</sub>N<sub>6</sub>O<sub>4</sub>.4CF<sub>3</sub>CO<sub>2</sub>H, C<sub>88</sub>H<sub>178</sub>N<sub>8</sub>O<sub>4</sub>S<sub>2</sub>.4CF<sub>3</sub>CO<sub>2</sub>H, C<sub>40</sub>H<sub>84</sub>NO<sub>3</sub>P.CF<sub>3</sub>CO<sub>2</sub>H, C<sub>50</sub>H<sub>103</sub>N<sub>7</sub>O<sub>3</sub>.4CF<sub>3</sub>CO<sub>2</sub>H,

30 C<sub>55</sub>H<sub>116</sub>N<sub>8</sub>O<sub>2</sub>.6CF<sub>3</sub>CO<sub>2</sub>H, C<sub>49</sub>H<sub>102</sub>N<sub>6</sub>O<sub>3</sub>.4CF<sub>3</sub>CO<sub>2</sub>H, C<sub>44</sub>H<sub>89</sub>N<sub>5</sub>O<sub>3</sub>.2CF<sub>3</sub>CO<sub>2</sub>H, C<sub>100</sub>H<sub>206</sub>N<sub>12</sub>O<sub>4</sub>S<sub>2</sub>.8CF<sub>3</sub>CO<sub>2</sub>H, C<sub>41</sub>H<sub>78</sub>NO<sub>8</sub>P) C<sub>162</sub>H<sub>330</sub>N<sub>22</sub>O<sub>9</sub>.13CF<sub>3</sub>CO<sub>2</sub>H, C<sub>43</sub>H<sub>88</sub>N<sub>4</sub>O<sub>2</sub>.2CF<sub>3</sub>CO<sub>2</sub>H, C<sub>43</sub>H<sub>88</sub>N<sub>4</sub>O<sub>3</sub>.2CF<sub>3</sub>CO<sub>2</sub>H, and (1-methyl-4-(1-octadec-9-enyl-nonadec-10-enylenyl) pyridinium chloride.

49. The method of claim 37, wherein:  
the nucleic acid molecule is exposed to an agent that increases contact between the  
nucleic acid molecule and the cell; and  
the cell is exposed to an agent that enhances permeability of the cell.
- 5 50. The method of claim 49, wherein the exposure of the cell to an agent that enhances  
permeability comprises applying ultrasound or electrical energy to the cell.
51. The method of claim 37, wherein the cell is a mammalian cell.
52. The method of claim 37, wherein the cell is a human cell.
53. The method of claim 37, wherein the cell is a synoviocyte.
- 10 54. The method of claim 37, wherein the cell is a fibroblast-like synoviocyte.
55. The method of claim 37, wherein the pre-selected phase is determined by a method  
comprising:  
introducing nucleic acid into cells at different phases;  
determining and comparing the efficiency of delivery and/or transfection of the  
15 nucleic acid into the cells at different phases; and  
selecting a phase at which the efficiency of delivery and/or transfection is increased  
relative to efficiency of delivery and/or transfection at other phases.
56. The method of claim 37, wherein the cell that is in a pre-selected phase is obtained by  
a method comprising:  
20 exposing one or more cells to a cell cycle arrest agent;  
exposing the one or more cells to conditions that permit cell cycling;  
selecting a cell that is in the pre-selected phase.
57. The method of claim 56, wherein the arrest agent is an anti-microtubule compound.
58. The method of claim 56, wherein the arrest agent is nocodazole or thymidine.
- 25 59. The method of claim 37 or 56, wherein the pre-selected phase cycle is G2/M.
60. The method of claim 56, wherein exposure of the one or more cells to an arrest agent  
results in arrest of the one or more cells in mitosis or G0/G1.
61. The method of claim 37, wherein the nuclear membrane of the cell is absent.
62. The method of claim 37, wherein a plurality of cells in a pre-selected phase is  
30 obtained and the plurality of cells is contacted with one or more nucleic acid molecules,  
whereby the nucleic acid molecule(s) is delivered into one or more cells, and one or more  
cells are introduced into the subject.

63. The method of claim 62, wherein the plurality of cells is in a synchronous population of cells.

64. The method of claim 63, wherein the efficiency of transfection of the nucleic acid molecule to cells in a synchronous population of cells is at least 1.5, at least 2, at least 2.5, at least 3, at least 3.5, at least 4, at least 4.5, at least 5, at least 5.5, at least 6, at least 6.5, at least 7, at least 7.5, at least 8, at least 8.5, at least 9, at least 9.5 or at least 10-fold greater than the efficiency of delivery of the nucleic acid molecule to cells in an asynchronous population of cells.

65. The method of claim 63, wherein the nucleic acid molecule is a chromosome or artificial chromosome and the percentage of cells comprising an intact delivered chromosome after contact of a synchronous population of cells with the chromosome or artificial chromosome is greater than the percentage of cells comprising an intact delivered chromosome after contact of an asynchronous population of cells with the chromosome or artificial chromosome.

66. The method of claim 65 wherein the artificial chromosome is an ACes.

67. A method for *ex vivo* gene therapy, comprising:  
contacting a cell with a chromosome or fragment thereof that is in the same or similar phase as the cell, whereby the chromosome or functional fragment thereof is delivered into the cell; and

introducing the cell into a subject.

68. The method of claim 67 wherein the chromosome is an artificial chromosome.

69. The method of claim 68, wherein the chromosome is an ACes.

70. A kit for delivering large nucleic acids into cells, comprising:

a delivery agent;

a cell cycle arrest agent; and

optionally instructions for delivering large nucleic acids into cells.

71. A method for selecting a host cell for receiving a large nucleic acid molecule, comprising:

introducing a large nucleic acid molecule into cells at different phases;

determining and comparing the efficiency of delivery and/or transfection of the nucleic acid into the cells at different phases; and

selecting a cell that is in a phase that provides for increased efficiency of delivery and/or transfection relative to the efficiency of delivery and/or transfection at other phases.

72. The method of claim 71, wherein the large nucleic acid molecule is labeled.
73. The method of claim 72, wherein the labelled cells are detected by flow cytometry, fluorimetry, cell imaging or fluorescence spectroscopy.
74. The method of claim 73, wherein the labelled cells are detected by flow cytometry.
- 5 75. The method of claim 73, wherein the label is iododeoxyuridine (IdU or IdUrd) or bromodeoxyuridine (BrdU).
76. The method of claim 73, wherein the large nucleic acid molecule is a chromosome or functional fragment thereof.
- 10 77. The method of claim 73, wherein the large nucleic acid molecule is an artificial chromosome.
78. The method of claim 77, wherein the artificial chromosome is an ACes.
79. The method of any of claims 1-33, 37-47 or 56, wherein the pre-selected phase of the cell is selected from the group consisting of G1, S, G2, G2/M, M and any of the stages of the M phase.
- 15 80. The method of claim 1, 23, 47, or 56, wherein the pre-selected phase of the cell cycle is not G0.
81. The method of any of claims 1-33, 37-66 and 71-78, wherein the large nucleic acid is associated with one or more proteins.
82. The method of any of claims 34-36 and 67-69, wherein the chromosome or functional 20 fragment thereof is associated with one or more proteins.

## SEQUENCE LISTING

<110> CHROMOS MOLECULAR SYSTEMS, INC.

DE JONG, Gary  
PERKINS, Edward  
TELENIUS, Adele  
MACDONALD, Neil

<120> METHODS FOR DELIVERING NUCLEIC ACID MOLECULES INTO CELLS AND ASSESSMENT THEREOF

<130> 82828-5

<140> To Be Assigned  
<141> 2003-04-30

<150> US 60/377547  
<151> 2002-05-01

<160> 13

<170> FastSEQ for Windows Version 4.0

<210> 1  
<211> 24  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Primer

<400> 1  
cgtccaggag cgaccatct tott

24

<210> 2  
<211> 24  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Primer

<400> 2  
atcgcgcttc tcgttgggt cttt

24

<210> 3  
<211> 30  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Primer

<400> 3  
aggactgggt ggcttccaac tccccagacac

30

<210> 4  
<211> 30  
<212> DNA  
<213> Artificial Sequence

<220>

<223> Primer

<400> 4  
agtttctcat tgctgcgcgc cagggttcagg

30

<210> 5  
<211> 22118

<212> DNA  
<213> *Mus musculus*

<400> 5		60
gaattccccct atccctaatac cagattgggt gaataacttg gtatagatgt ttgtgcatta		120
aaaaccctgt agatcttca ctctaggta ctgttcagca ctggAACCTG aattgtggcc		180
ctgagtgata ggtcctggga catatgcagt tctgcacaga cagacagaca gacagacaga		240
cagacagaca gacagacgtt acaaacaac acgttgagcc gtgtGCCAAC acacacacaa		300
acaccactct ggcataatt attgaggacg ttgattttt attctgtgtt tgtgagtctg		360
tctgtctgtc tgtctgtctg tctgtctgtc tatcaaacc aaaaaacca aacaattatg		420
cctgcctgcc tgccctgcctg cctacacaga gaaatgattt cttcaatcaa tctaaaacga		480
cctcctaagt ttgcctttt tctcttctt tacctttt tttttctt ttttcttct		540
tccttccttc ctcccttcct tccttcctt ctttcttctt ttcttctt ctactttct		600
ttcttcctt ctacatittt ttctttcat acatagttt ttagttaag catccctgac		660
tgtcttgaag acactttgtt ggcctcaatc ctgttaagagc cttccctgc ttttcaaattg		720
ctggcatgaa ttttgttacact cactatgacc agcttagtct tcaagtctg gttactggaa		780
aggagttcca agaagactgg ttatattttt catttattat tgcattttaa taaaaattta		840
atttcaccaa aagaatttag actgaccaat tcagagtctg ccgtttaaaa gcataaggaa		900
aaagtaggag aaaaacgtga ggctgtctgt ggatggtcga ggctgttta gggagcctcg		960
tcaccattct gcaacttgcaa accgggccac tagaaccgg tgaagggaga accaaaacgg		1020
acctggaaac aataggtcac atgaaggcca gcccacttca tcttgggtt cgggagttca		1080
gttagcagac aagatggctg ccatgcacat ttgtctttc agcttggta ggtcaaaatg		1140
caaccgagtc acagaacaag gaagtataca cagttagttc caggtcagcc agagtttaca		1200
cagagaaacc acatcttga aaaaacaaaa aataaaattt aataaaatata atttttttt		1260
ttttttttttt ccgggagtga tggcgcattt ctttatccc agctctttc aggagagat		1320
gggaggattt ctgagtttga ggcacccctt gtctgtttaa tgagttccag gacagtcagg		1380
gctatacaga gaaaccctgt ttgtttttt aaactaaattt aaactaaact aaactaaaaa		1440
aatataaaat aaaaattttt aagaattttt aaaaactaca gaaatcaaacc ataagccac		1500
gagatggcaa gtaactgcaa tcatacgaaa aatattatac acacacacac acacagactc		1560
tgtcataaaa tccaatgtgc cttcatgtat atcaattttc gatagtcaat aataactagaa		1620
gaatcatatg tctggaaaataa aagccagaa cttttctgc ttttttttcc ttttgcucca		1680
agatagggtt tctctcatttgc tatccctggc atccctgc ggaacttcc ttgttaggtt		1740
ggtagcctca aactcgagaa ggtccctctt gcctgcctgc ctgcctgcctt gcctgcctgc		1800
ctgcctgcctt gcctgcctca cttctctgc caccacaca accgagtcga acctaggatc		1860
tttattttctt tctcttcttctt ttctttctt ttctttctt ctttcttctt ttctttctt		1920
ttttttttttt ttcttatttca attgttttca aatgttaatgt ttgtttgtt ctctatctgc		1980
tgcctataagg cctgcatttcc aggagggc aacagaacctt aggagaaacc accatgcac		2040
tcctgagaat aagtggaaaaa acaacaaaaa aaggaaattt taatcacata gaatgttagat		2100
atatgccgag gctgtcagag tgcttttaa ggcttagtgt aagtaatgaa aattgttg		2160
tgtcttttat ccaaaccacag aagagaggtt gtcggccctg catgtctgtt gtctgcatt		2220
agaccaggct ggccttgaac acattaaatct gtctgcctt gttttccctaa tgctgcatt		2280
aaaggcatgt gccaccactg cccggactga tttttttttt tttttttttt tgaaaaatac		2340
ctttcttctt tttctcttctt ctctttcttctt ttctttcttctt ttctttcttctt		2400
tttctttttt tttttttttt ttttttttaa aatttgccta aggttaaagg tttttttttt		2460
aattgcctca gctctgcctt aatttctttttt aaaaaaaaaa aaacaaaaaaa aaaacaaaaa		2520
cagtatgtat gtatgtat ttagaagaaa tactaatcca ttaataactc ttttttctta		2580
aaattcatgt cattcttggt ccacaaaatgt agttccagga cttaccagag aaaccctgt		2640
ttcaaaatttca tttttttttt tttttttttt aatttgccta aggttaaagg tttttttttt		2700
ctacacagaa aaaccatatac tcagaaaaaaa aaaaagttcc aaacacacac acacacacac		2760
acacacacac acacacacac acacacacac acacacacag cgcggccgg cgatgagggg		2820
aaatcgatggcc taaaataat atttttctgg ccaaagtggaa agcaaatcactatgtaaaggg		

tactcctaga	aaaaataaaat	acaaacgggc	tttttaatca	ttccagcact	gttttaattt	2880
aactctgaat	ttagtcttgg	aaaagggggc	gggtgtgggt	gagttagggc	gagcgagcag	2940
acggcgggc	gggcgggtga	gtggccggcg	gcggtggcag	cgagcaccaag	aaaacaacaa	3000
accccaagcg	gttagagtgtt	ttaaaaatga	gacctaattg	ttgttggaaacg	gaggtcggcg	3060
ccaccctct	cttccactgc	ttagatgctc	cottcccctt	actgtgctcc	tttcccctaa	3120
ctgtgcctaa	ctgtgcctgt	tccctcaccc	cgctgattcg	ccagcgaact	acttgactt	3180
caagaacat	tttgctgtt	tccaccgctc	cctgtcatac	tttcgttttt	gggtgcccga	3240
gtctagcccg	ttcgctatgt	tcggccggga	cgatggggac	cgtttgc	actcgggaga	3300
agtggtgggt	gggtacgctg	ctccgtcgtg	cgtgcgtgag	tgccggaaacc	tgagctcggt	3360
agaccctccg	gagagacaga	atgagtgaat	gaatgtggcg	gcgcgtgacg	gatctgtatt	3420
ggtttgtatg	gttgatcgag	accattgtcg	ggcgacacact	agtggtgaca	agttcggga	3480
acgtctccagg	cctctcagg	ttgtgacaca	ggagagggaa	gtgcctgtgg	tgaggcgacc	3540
agggtgacag	gaggccgggc	aagcaggccg	gagcgtctcg	gagatgggtt	cgtgtttaag	3600
gacggctctc	aacaaggagg	tcgtacagg	agatggccaa	agcagaccga	gttgcgttac	3660
gcccttttgg	aaaaaatgt	agggttgggt	gcaacgttac	taggtgcacc	agaaggctt	3720
agtcctaccc	ccccccccc	ttttttttt	tttctccag	aagccctctc	ttgtccccgt	3780
caccgggggc	accgtacatc	tgaggccgag	aggacgcgt	ggggccggct	tccaagccgg	3840
tgtggctcgg	ccagctggcg	tttcgggtct	ttttttttt	ttttttttt	ttttctcca	3900
gaaggccttgt	ctgtgcgtgt	caccgggggc	gctgtacttc	tgaggccgag	aggacgcgt	3960
ggccccccgg	ttccaagccg	gtgtggctcg	gccagctgga	gcttcgggtc	ttttttttt	4020
ttttttttt	tttttttctc	cagaagcctt	gtctgtcgct	gtcaccgggg	gcgtgtact	4080
tctgaggccg	agaggacgcg	atgggtcgcc	ttccaagccg	atgtggccgg	gccagctgga	4140
gcttcgggtt	ttttttttt	ctccagaagc	cctctcttgt	ccccgtcacc	ggggccgtcg	4200
tactctctgag	gccgagagga	cgtgatgggc	ccgggttcca	ggcggatgtc	gcccggtcag	4260
ctggagctt	ggatctttt	ttttttttt	cctccagaag	ccctcttgc	tcccgtcacc	4320
cgggggcacc	ttacatctga	gggcgagagg	acgtatggg	tccggcttcc	aagccgtatgt	4380
ggcggggcca	gctggagctt	cggtttttt	tttttcctc	cagaagccct	ctttgttccc	4440
cgtcaccggg	ggcgtctgtac	ttctgaggcc	gagaggacgt	gatggggcccg	gttccaggc	4500
gatgtcggcc	cggtcagctg	gagctttgga	tcatttttt	ttttccctcc	agaagccctc	4560
tcttgccccc	gtcaccgggg	gcaccgtaca	tctgaggccg	agaggacacg	atgggcgtgt	4620
cttccaagcc	gatgtggccc	ggccagctgg	agcttcgggt	ttttttttt	tttttttctc	4680
cagaagccct	gtctgtcgct	gtcaccgggg	gcgtgtact	tctgaggccg	agaggacgcg	4740
atggggcccg	tttccaagcc	gggtgtggctc	ggccagctgg	agcttcgggt	ttttttttt	4800
ttttttttt	ttcctccaga	aaccttgc	gtcgtgtca	ccggggccgc	tttgacttct	4860
gatgccgaga	ggacgcgtat	ggccgtctt	ccaggccgt	gtggcccggt	cagctggagc	4920
tttggatctt	ttttttttt	tttcctcca	gaagccctt	ttgttccccc	tcacccgggg	4980
caccttacat	ctgaggccct	gaggacacga	tggccgggg	ttccaggccg	atgtggcccg	5040
gtcagcttgg	gtttttttt	tttcttcca	gaagccctt	tttcccccgtc	5100	
accgggtggca	ctgtacatct	gaggcggaga	ggacattatg	ggccgggtt	ccaatccgt	5160
gtggcccggt	cagctggagc	tttggatctt	atttttttt	taatttttt	ttccagaagc	5220
cctcttgc	ctgtcaccgg	tggcacgta	catctgaggc	cgagaggaca	ttatggggcc	5280
ggcttccagg	ccgatgtggc	ccggtcagct	ggagctttgg	atctttttt	tttttttct	5340
ttttccctcc	agaagccctc	tctgtccctg	tcacccgggg	ccctgtacgt	ctgaggccga	5400
gggaaagcta	tgggcgggt	tttcttcat	tgacctgtcg	gtcttatcag	ttctccgggt	5460
tgtcagggtc	gaccagtgt	tcctttgagg	tccggtttct	ttcggttatgg	ggtcattttt	5520
gggccaccc	cccaggatgt	acttccaggc	gtcggtgtc	gcctgtact	ttctccctg	5580
tctctttat	gtttgtgtac	ttttctatct	gttcttattg	gacctggaga	taggtactga	5640
cacgctgtcc	tttccctatt	aacactaaag	gacactataa	agagaccctt	tcgatattaag	5700
gtcttttgc	ttgtccagcc	tattttttt	actggcttgg	gtctgtcgcg	gtccctgaag	5760
ctgtccccc	gccacgttc	ctgtttccc	gggttgcgt	cttgcgtgt	cttgcgtgt	5820
gcagcttgg	acaactgggc	gtgtgactt	tgctgcgtgt	cagacgtttt	tcccgttcc	5880
cccgagggtt	cgttgcaca	cctgtccccc	ttgaatgggt	ggagccagct	gtgggttgg	5940
gccacccat	ttcggctc	ttttttttt	tttttttctc	ttggagatccc	gaacccccc	6000
tctttctct	tccggctt	tcttccacat	gcctcccgag	tgcatattctt	tttgtttttt	6060
ttctttttt	ttttttttt	ttggggaggt	ggagagatccc	gagacttca	ctccctgtctg	6120
tggtgtccaa	gtgttcatgc	cacgtgcctc	ccgagtgcac	ttttttttgt	ggcagtcgct	6180
cgttgtttc	tcttgttctg	tgtctggccc	tatcagtaac	tgtcttgc	cgcgtgttaag	6240
acattccat	ctcgcttgtt	tctcccgatt	gcccgtcggt	gctcaactt	agatcgatgt	6300
ggtgctccgg	agtctcttc	ggccaggggc	caagccgcgc	caggcgaggg	acggacattc	6360
atggcgaatg	gcggccgctc	ttctcggttct	gccagcgccc	cctcgctct	ccacccccc	6420

cgtctgccgg	tggtgtgtgg	aaggcagggg	tgccgccttc	cggccccgacg	ctgccccgcg	6480
cgcacttttc	tcagtggttc	gcgtggtc	tgtggatgtg	tgaggcgccc	ggttgtgccc	6540
tcacgtgttt	cacttggtc	gtgtctcgct	tgaccatgtt	cccagagtcg	gtggatgtgg	6600
ccgggtggcgt	tgcataccct	tcccgtctgg	tgtgtgcacg	cgctgtttct	tgttaagcgtc	6660
gagggtgtcc	tggagcggttc	cagggttgc	tccttaggtgc	ctgtttctga	gctgtgtgg	6720
gcgcgtccca	ttccctgggt	tgccctccgt	gctccgtctg	gctgtgtgcc	ttcccggttg	6780
tgtctgagaa	gcccgtgaga	gggggggtcga	ggagagaagg	aggggcaaga	ccccccctct	6840
tctgtcggtt	aggcgcacac	cccgcgacta	gtacgcctgt	gcgttagggct	ggtgtcgagc	6900
ggtcgcggcgt	gggggtggaa	agtttctcga	gagactcatt	gtttttccgt	ggggagcttt	6960
gagaggcctg	gctttcgggg	gggaccgggt	gcagggcttc	ccctgtccgc	ggatgtctag	7020
aatgccttgc	gaagagaacc	ttcctgttc	cgcaaaaa	cccgcgccgt	cccccgctg	7080
ttggtcttct	ggtttccctg	tgtgtctgc	gcatgcattc	tctctcggtg	gccggggctc	7140
gtcggggttt	tgggtccgtc	cgcgcctcag	tgagaaagt	tccttctata	gctatcttc	7200
ggaaagggtg	cgggccttctt	acgggtctcga	ggggctcttc	ccgaatggtc	ccctggaggg	7260
ctcgccccct	gaccgcctcc	cgcgccgcga	gcgttgc	tctcgctcac	cgccggccgc	7320
ggcctccccc	ctccgagttc	ggggagggtat	cacgcggggc	agagcctgtc	tgtgtctcg	7380
cggttgcgtc	ggagcatgtg	gtcggcttg	tgtgttggt	ggctggggag	agggctccgt	7440
gcacaccccg	gcgtgcgcgt	actttcttc	cctctgagg	gccgcgtgc	ggacgggggt	7500
tggtagggc	acgggtggct	cccggtccc	caccgtctt	cccggtccctc	accgtgcct	7560
tccgtcgct	gcgtccctct	cgctcgctc	cacgactttg	gccgctcccg	cgacggccgc	7620
ctgcgcgcg	cgtggtgcgt	gtgtgtgt	tctcggtctg	tgtgttggt	tcgcctcgcc	7680
ccccctttcc	cgcggcagcg	ttcccacggc	tggcgaatc	gcgggagtc	tccttccct	7740
cctcggggtc	gagagggtcc	gtgtctggcg	ttgattgatc	tcgcctctgg	ggacgggacc	7800
gttctgtgg	agaacggctg	ttggccgcgt	ccggcgccac	gtcgacgtg	gggaccact	7860
gccgcgcggg	ggtcttcgtc	ggtaggcata	gtgtgtcg	catcggtctc	tctctcggt	7920
cggtgtcgcc	tcctcggtc	cccggggggc	cgtgtgttt	cggtcgct	cgccgtcgca	7980
gttgtgggtt	gactgtcag	gggagtggtg	cagtgtgatt	cccgccgtt	ttgcctcg	8040
tgccctgacc	ggtccgacgc	ccgagcggtc	tctcggtccc	ttgtgaggac	cccttccgg	8100
gaggggcccc	tttggccgc	ccttgcgtc	gtcgccggcc	ctgttctgc	tgtgtcggt	8160
ccccctcccc	gctcgccca	gcccgttctt	tttctctct	ccccccctct	cctctgactg	8220
acccgtggcc	gtgtgtcg	accccccga	tggggcgcc	ccggcaccta	cgcgccgg	8280
cggtcacccg	ggttttgggg	ggggcccgag	ggtaagaaa	gtcggtcg	cgggccggag	8340
gagctgtgtt	ttggagggcg	tcccggcccc	gcccgttgg	cggtgttttg	cgccgttttg	8400
gagagggctg	cgtcgaggg	aaaaagggtt	cccccgcagg	gcaaaggaa	agaggctagc	8460
agtggtcatt	gtccgcacgg	tgtgtgggt	tgttggccga	gtgcgtctg	gggggctcg	8520
ccggccctgt	cgtccgtcg	gaaggcgcgt	gttggggct	ccggagatgc	cgagggtgg	8580
accctggccgg	tgggattaac	cccgcgccgc	tgttcccggt	tggcggtgg	ggctccgg	8640
gatgtctacc	tccctctccc	cgagggtctc	ggccttctcc	gecgccgtc	tcgccttcc	8700
cctcggttct	ccctctcg	gggttcaagt	cgctcgta	cctcccttc	tccgtccctc	8760
catctctcgc	gcaatggcgc	cccccgaat	cacgggtgg	tgcgttcc	cctccgttc	8820
tcgcccgggg	ctggcgctg	tccggtctc	cctcccgac	ccccgttggc	gtggtcttct	8880
ctcgccggct	tcgcccact	ctgggttc	ccggagggtc	agggggcttc	ccggttcccc	8940
gacgttgcgc	ctcgctgt	tgtgtttgg	ggggcccg	tcggccctcc	gcccggccgt	9000
gagccctgtc	cgcacccggc	gggtgtcggt	ttcgcggc	ggtcagtgg	ccctgtggcg	9060
tgtgtcgct	cggagcggt	tccgtcg	ggccgttaga	cgccgggtgc	gcccggctcc	9120
gacgggtggc	ctatccaggg	ctcgcccccg	ccgacccccc	cctccgtc	ccgggtgg	9180
tcgttgggt	ggggaggtaa	tgggtctacc	gtcattccc	tcccggtgg	tttgactgtc	9240
tcgcccgggt	cgcgttctc	tttccgcca	ccccacggc	aacccaccac	cctgtctcc	9300
cggcccggt	cgggtcgacgt	tccggcttc	ccgatgccg	ggggttgggg	atttgtccg	9360
gggacggagg	ggagagcggt	taagagaggt	gtcgagagc	tgtccgggg	cgacgtcg	9420
gttggctttg	cccggtcggt	gtgtcg	acgggtttt	tcggaccccg	acggggtcgg	9480
tccggccca	tgcactctcc	cggtccgc	gagcgccgc	ccggctacc	cccggtttgt	9540
cctcccgcc	ggctctccgc	cgccggcc	tcctctct	ctctcg	ctctgtcccg	9600
cctggtctg	tccacccccc	gacgctccgc	tcgcgttcc	ttacctgg	gatcctgc	9660
gtagcatat	gttgcgtca	aagattaagc	catgcattgc	taagta	cgccgggtac	9720
agtgaaactg	cgaatggctc	attaaatcg	ttatggttcc	tttggtcg	cgctcctctc	9780
ctacttgat	aactgtggta	attctagagc	taatacatgc	cgacggccgc	tgacccccc	9840
tcccgggggg	ggatgcgtc	atttatcaga	tcaaaaccaa	cccggtgagc	tccctcccg	9900
ctccggccgg	gggtcgccg	ccggcggttt	ggtgactcta	gataacctcg	ggccgatcgc	9960
acgcccccccg	tggcgccgac	gaccattcg	aacgtctgcc	ctatcaactt	tcgatggtag	10020

tccgcgtgcc	taccatggtg	accacgggtg	acgggaaatc	agggttcgtat	tccggagagg	10080
gagcctgaga	aacggctacc	acatccaagg	aaggcagcag	gcgcgcaaat	tacccactcc	10140
cgacccgggg	aggtagtgac	aaaaaataac	aatacaggac	tcttcgagg	ccctgttaatt	10200
ggaatgagtc	cactttaaat	ccttaacga	ggatccattg	gagggcaagt	ctggtgccag	10260
cagccgcgtt	aattccagct	ccaatagcgt	atattaaagt	tgctgcagtt	aaaaagctcg	10320
tagttggatc	ttgggagcgg	gcgggcccgtc	cgccgcgagg	cgagtcaaccg	ccctgtccccg	10380
ccccttgcct	ctcggccccc	cctcgatgt	cttagctgag	tgtcccgccgg	ggcccgaagc	10440
gtttactttg	aaaaaattag	agtgttcaa	gcagggccga	gccgccttgg	taccgcagct	10500
aggaataatg	gaataggacc	gcgggtctat	tttggtggtt	ttcggaaactg	aggccatgtat	10560
taagagggac	ggccgggggc	attcgatattg	cgccgcctaga	ggtaaaatcc	ttggaccggc	10620
gcaagacgga	ccagagcgaa	agcatttgcc	aagaatgttt	tcattaatca	agaacgaaag	10680
tcggaggttc	gaagacgatc	agataccgtc	gtatggccga	ccataaacga	tgccgactgg	10740
cgatgcggcg	gcgttattcc	catgaccgc	cgggcagtt	ccggggaaacc	aaagtctttg	10800
gttccgggg	ggagtatggt	tgcaaagctg	aaacttaaaag	gaatttgacgg	aagggcacca	10860
ccaggagtgg	gcctgcggct	taatttgact	caacacggga	aacctcaccc	ggcccgacca	10920
cgacacggat	tgacagattt	atagctttt	ctcgattccg	tgggtgggtt	tgcatggccg	10980
ttcttagttg	gtggagcgt	ttgtctggtt	aattccogata	acgaacgaga	ctctggcatg	11040
ctaacttagt	acgcgacccc	cgagcggctc	gcgtccccc	acttctttaga	gggacaagtg	11100
gcgttcagcc	acccagaggat	gagcaataac	aggctctgt	tgccctttaga	tgtccggggc	11160
tgcacgcgc	ctacactgac	tggctcagcg	tgtgcctacc	ctgcgcgggc	aggcgcgggt	11220
aaccctgttga	acccattcg	tgatggggat	cggggattgc	aattattttcc	catgaacgag	11280
gaattcccaag	taagtgcggg	tcataagctt	gcgttgatta	agtcctgtcc	cttgcacac	11340
accggccgcgtc	gctactaccg	attggatggt	ttagtgaggc	cctcggatcg	gccccggccgg	11400
gttcggccca	cgggcctggc	ggagcgtctg	gaagacggtc	gaacttgact	atctagagga	11460
agtaaaaatgc	gtaacaagg	ttccgttaggt	gaacctgcgg	aaggatcatt	aaacgggaga	11520
ctgtggagga	gcggcggcgt	ggccgcgtt	cccgcttgc	tgtgtgttcc	cgccgggagg	11580
cgcgtgcgtc	ccgggtcccg	tcgcccgcgt	gtggagcgg	gtgtctggag	tgaggtgaga	11640
gaagggggtgg	gtgggttcgg	tctgggtccg	tctgggaccg	cctccgattt	cccttcccccc	11700
tccctcttcc	ctcgccggc	tctgaccc	ccaccctacc	gccccggcgg	ctgctcgccg	11760
gcgttgcgc	tctttccgt	ccggcttcc	cgtgtctacg	aggggcgtt	cgtcgatc	11820
gttttttgc	ccgtcccccgg	ggcgttcgg	cgtcggggcg	cgcgtttgc	tctccggca	11880
ccatccccg	ccggggctct	ggcttttcta	cgttggctgg	ggcgggttgc	gcgtgtgggg	11940
ggatgtgagt	gtcgctgt	ggctcgccg	tcccgatgc	acgttttct	ggcctcgccgt	12000
gtcttcccg	ctcctgtccc	gggtacccat	ctgtcgctt	ccggcggcga	gtttaagga	12060
ccccgggggg	gtcgccctgc	cgcccccagg	gtcgggggcc	gttggggccc	gtagggaaat	12120
cggtcggtcg	ggcgctctc	cctcagactc	catgaccctc	ctccccccgc	tgccgcccgtt	12180
cccgaggcgg	cggtcggt	gggggggttga	tgtctggagc	ccctctggc	gcccgtgggg	12240
cccgaccgc	gcccggcgt	tgcccgattt	ccgggggtcg	gtctgtcgg	tgccgggtcg	12300
gggttcccg	gtcggtcccg	tgtttttccg	ctcccgaccc	ttttttttcc	ctccccccca	12360
cacgtgtotc	gtttcggtcc	tgctggccgg	cctgaggct	ccctctggtc	cattgttct	12420
cctctcttc	cgggagagg	aggggcgggtt	tctttgggg	actgtgcgt	cgtcagcacc	12480
cgtgagttcg	ctcacacccg	aaataccat	acactctt	gccccggatc	actcggtctcg	12540
tgcgtcgatg	aagaacgcag	ctagctgcg	gaattaatgt	gaattgcagg	acacattgtat	12600
catcgacact	tcgaacgcac	ttgcggcccc	gggttcctcc	ccgggctacg	cctgtctgag	12660
cgtcggttga	cgatcaatcg	cgtcaccgc	tgccgtgggt	gctgcgcggc	tgggagtttgc	12720
ctcgcagggc	caaaaaaaaa	acccgggtcg	ggccctccgt	ctcccgaaat	tcaagacgtgt	12780
gggcgggttgc	cggtgtggcg	cgccgcggcg	cggtgtggcg	ctgggtctcc	cccgcgcatc	12840
cgcgtcgcg	gtttttcccg	gtcccgccgt	tccgccttc	ggccgtgcac	cccggtctcg	12900
gcctcgccgc	ggcgccctcc	ggaccgcgtc	ctcaccagtc	tttctcggtc	ccgtgccccg	12960
tgggaaccca	cccgcccccc	tgggcggccg	gggggtggcg	cgccgcgcac	tgtctcggtc	13020
gagggtggcg	ttggagggttgc	tgccgtcgcc	gagggtggtg	tcgggtccct	gccccggccgg	13080
ggttgtcggt	gtggcggtcg	acgaggggccg	gtccgtcgcc	tgccgtgggtt	gtctgtgtgt	13140
gtttgggtct	tgcgctgggg	gaggcggttgc	cgaccgcgtc	cggggttggc	gccccggccccc	13200
ggcgcggcgc	accctccggc	ttgtgtggag	ggagagcggag	ggcgagaaatc	gagagagggt	13260
gtatccccgg	tggcggtcg	agggagggtt	tgccgtcccg	cgccgcgtcc	tcctcccttc	13320
cctcggtggg	cgccttcgcg	ccgcacgcgg	ccgtctagggg	cggtcgcccc	ccgtggccccc	13380
cgtggcttct	cttcgtctcc	gtttctctt	caccggccgc	gtaccgcgtc	cgccgcggccgc	13440
cccgcggtac	ccgcggcggtc	cgtgcggcga	tgcgagtcac	ccccgggtgt	tgcgagttcg	13500
gggaggggaga	gggcctcgct	gaccgcgtc	gtcccgccgtt	ccctgggggg	gaccggcgt	13560
ctgtgggtcg	tgcgtcccg	gggttgcgt	ttagtaagat	cctccaccccc	cgccgcggcc	13620

ccctcccgcc	ggccttcgg	gaccccccgt	agacggttcg	ccggctcg	ctcccggtcc	13680
gcgggtgcc	gtctcttcc	cgcggccctc	ctcgctct	tctcccgcg	gctggggcg	13740
tgtccccct	ttctgaccgc	gacctcagat	cagacgtggc	gaccgcgt	attnaagcat	13800
attagtca	ggagaaaaag	aaactaacca	ggattccctc	agtaacggcg	agtgaacagg	13860
gaagagccca	gcgcgaatc	ccgcggcg	gtcgccgt	gggaaatgt	gcgtacggaa	13920
gaccactcc	ccggccggc	tcgtgggg	cccaagtcc	tctgatcg	gcccagcccg	13980
tggacggtgt	gaggccggta	gcccccccg	cgccgggg	tcgggtctt	ccggagtcgg	14040
gttgcttggg	aatgcagccc	aaagcggtt	gtaaactcca	tctaaggct	aataccggca	14100
cgagaccgat	agtcaacaag	taccgttaagg	gaaagttaa	aagaatttt	aagagagagt	14160
tcaagagggc	gtgaaaccgt	taagaggtaa	acgggtggg	tccgcgcgt	ccgccccggag	14220
gattcaaccc	ggcggcg	gtccggcc	gcccgtgt	ccggcggt	ctttcccgct	14280
ccccgttct	cccgacccct	ccacccgc	gtcg	tcttccttcc	cggtccggc	14340
gcctccggcg	gcggg	gggggtgt	gtgtggcg	cgccgggg	gccccgggt	14400
gggtcgccgg	gggacccccc	ccggccggcg	accggcc	gcccggcg	cttccaccgt	14460
ggcggtgcc	cgcgacccgg	tccgggacgg	ccggaaaggc	ccgtgggg	aggtggctcg	14520
ggggggggcg	cgcgtct	ggegcggcc	accacccac	cccgagtgtt	acagccctcc	14580
gcgcggcgtt	tcgcca	ccggggccga	ggaagccaga	tacccgtc	cgcgctctcc	14640
ctctccccc	gtccgctcc	ccggccggcg	tgggggtgg	ggccggcc	ccctcccac	14700
ggcgaccccg	ctctccacc	ccctccgtc	gcctctct	ggcccggt	ggggccgggg	14760
cgactgtcc	ccagtgcgc	ccggcg	tcgcgg	gggtccccgg	gggaccgtcg	14820
gtca	tcccgacgaa	gccgagc	cggg	ggcgatgtcg	gtacccacc	14880
cgaccgtct	tgaaaacacgg	accaaggagt	cta	cgcgagt	gggtcg	14940
gaaagccccc	gtggcgca	taaggtgaag	ggccccc	ggggcccga	gttggatcc	15000
cgaggcctt	ccagtccg	gagggcgcac	cac	tctcgg	cgcccccggg	15060
aggtggagca	cgagcgtac	cgtaggacc	cgaaagatgg	tgaactat	ttggcaggg	15120
cgaagccaga	ggaaactct	gtggagg	gtagcg	tgacgt	atcggtcg	15180
cgaccgttgt	atagggcga	aagacta	taatc	gtagctgtt	ccctccga	15240
tttccct	tagacttgc	gtctcg	ccgac	cagttt	cgtaaagcg	15300
aatgattaga	ggtcttggg	ccgaaacat	ctcaac	totcaaa	taatggta	15360
agaagccccc	ctcg	tggagccgg	cgtgg	gagtgc	tggccactt	15420
ttggtaagca	gaactggc	tgccggat	accgaa	gggtta	gcccgt	15480
gacgctatc	agacccaga	aaaggttt	gtt	acagcagg	gttggccat	15540
gaagtccgaa	tccgta	agtgt	aactc	ccgaa	tagccctgaa	15600
aatggatggc	gtcg	ggggccata	cccgg	gccc	gacggaa	15660
ggacgggagc	ggccgc	gtcg	cg	cg	ggggcccgt	15720
ccccgcctc	ccctccg	gcccgtt	cccc	gtcg	ggggccat	15780
cgccgcacg	agt	ccgtcg	gagc	gc	cgggccgg	15840
tggagccg	gcag	atctt	tt	tatt	aaac	15900
aggccga	gtt	ggatgt	tt	tt	tttt	15960
agagatggc	gagt	ccatgt	gtt	at	tttt	16020
cgaaagg	tcgg	atccc	gtt	cc	tttt	16080
cagtgcgt	acg	tcccg	ggaa	gg	tttt	16140
tttctt	ttt	cgcc	ggaa	cc	tttt	16200
ttggaa	tcg	ggcc	ttcc	cc	tttt	16260
ggggag	tgt	ccgc	gggt	cc	tttt	16320
gaacagcc	tgg	ccgc	gggt	cc	tttt	16380
acttcgggat	aagg	tcaat	gggt	cc	tttt	16440
ctggcg	ccgc	gggt	gggt	cc	tttt	16500
cccg	cccg	ccgc	gggt	cc	tttt	16560
cgtcg	ac	cc	gggt	cc	tttt	16620
cgccgg	gg	cc	gggt	cc	tttt	16680
acc	cc	gg	gggt	cc	tttt	16740
ctctgg	gg	gg	gggt	cc	tttt	16800
gttc	gg	gg	gggt	cc	tttt	16860
gttc	gg	gg	gggt	cc	tttt	16920
cccgat	cc	gg	gggt	cc	tttt	16980
gggg	cc	gg	gggt	cc	tttt	17040
cgccgg	cc	gg	gggt	cc	tttt	17100
gaatcc	gtt	aa	gggt	cc	tttt	17160
tgtattt	ccc	aa	gggt	cc	tttt	17220
acggcg	ta	aa	gggt	cc	tttt	

cgcgcatgaa	tggatgaacg	agattcccac	tgtccctacc	tactatccag	cgaaccaca	17280
gccaaggaaa	cgggcttggc	gaaatcagcg	gggaaagaag	accctgtta	gcttgactct	17340
agtctggcac	ggtgaagaga	catgagaggt	gtagaataag	tgggaggccc	ccggcgcccc	17400
gccccgtcct	cgcgtcggg	tcggggcacg	ccggcctcgc	ggccgcgg	tgaatacca	17460
ctactctcat	cgtttttca	ctgaccgggt	gaggcggggg	ggcgagcccc	gaggggctct	17520
cgcttctggc	gccaagcgtc	cgtcccgcbc	gtgcgggccc	gfcgcaccgg	ctccggggac	17580
agtgcgcagg	ggggagttt	actggggcgg	tacacctgtc	aaacggtaac	gcaggtgtcc	17640
taaggcgagc	tcagggagga	cagaaaccc	ccgtggagca	gaaggcggaa	agctcggttg	17700
atcttgattt	tcagtgacaa	tacagacccgt	gaaagcgggg	cctcacgatc	cttgcgtac	17760
tttgggtttt	aagcaggagg	tgtcagaaaa	gttaccacag	ggataactgg	cttgcgtccgg	17820
ccaagcggtc	atagcgacgt	cgctttttga	tccttcgtat	tcggcttc	ctatcattgt	17880
gaagcagaat	tcaccaagcg	ttggattttt	caccactaa	tagggaaacgt	gagctgggtt	17940
tagaccgtcg	tgagacaggt	tagttttacc	ctactgtat	tgtgttgg	ccatggtaat	18000
cctgctcagt	acgagaggaa	ccgcagggtt	agacatttg	tgtatgtgt	tggctgagga	18060
gccaatgggg	cgaacttacc	atctgtggg	ttatgactga	acgcctctaa	gtcagaatcc	18120
gccccagcgg	aacgatacgg	cagcggcggaa	ggagcctcgg	ttggcccccgg	atagccgggt	18180
ccccgtccgt	cccgctcggg	ggggcccccc	cgtcgcccc	cgccggcggc	gggtctcccc	18240
cgccggggcg	tcgggaccgg	ggtccgggtc	ggagagccgt	tcgtcttgg	aaacgggggt	18300
cgccgggaaa	ggggggccgc	ctctcgccc	tcacgttga	cgacgttcg	tgtggAACCT	18360
gfcgcataac	cattcgttaga	cgacgttgc	ctgggtcggg	gtttcgtagc	tagcagagca	18420
gtccccctcg	tgcgtatctat	tgaaagtca	ccctcgacac	aagggtttgt	ctctgcgggc	18480
tttcccgctg	cacgccccgt	cgctcgacg	cgaccgtgtc	gccgcgggg	cgtcacgggg	18540
ggggtegcct	cggccccccgc	gccccgggt	gaacgaccgt	gtgggtttt	ggggggggat	18600
cgtcttcctcc	tcgcgtctcc	gaggacgggt	cggttcttt	tcccttccg	tcgtctctcc	18660
ttgggtgtgg	agcctcgtgc	cgtcgaccc	gccccctgtcc	gtcgctgtcc	gcccagcccc	18720
cttgccttcc	ggccttggcc	aagccggagg	gcccgggggg	ggatcggcg	gcccggcgca	18780
ccgcggcgcg	gtgacgcacg	gtgggatccc	catctcgcc	cggtccgtc	ggacggccgg	18840
gttggagggg	cgggaggggt	ttttccctgt	aacgcccgt	tcggcgccag	gcctctggcg	18900
gccccgggggg	cgctctctcc	gcccggat	ccccactccc	gccccctctc	ttcgcgccgg	18960
gccccggcga	cgtcgatcg	agggggaggat	gtcgccgtgt	ggaggcggag	agggtccggc	19020
gccccggctc	ttccatTTT	tcccccggaa	tttcggaggt	cgaccgtac	tccggggcgcac	19080
actttgtttt	ttttttttcc	cccgatgtg	gagggtcgacc	agatgtccg	aagtgtcccc	19140
cccccccccc	ccccccggcg	cggagcggcg	ggggccactct	ggactctttt	tttttttttt	19200
tttttttttt	ttaaattcc	ggaaccttta	gtcgaccag	ttgtccgtct	tttactcc	19260
catataggtc	gaccgtact	ccgggtggta	ctttgtcttt	ttctgaaaat	cccagagggtc	19320
gaccagat	ccgaaagtcc	tctctttccc	tttactcttc	cccacagcg	ttctcttttt	19380
tttttttttt	tttgggtgtgc	cttttttga	tttatataca	tgtaaatagt	gtgtacgttt	19440
atataacttat	aggaggaggt	cgaccgtac	tccggggcgcac	actttgtttt	tttttttttt	19500
tccaccgtat	atggagggtcg	accagatgtc	cgaaagtgtc	ccgtcccccc	cctccccccccc	19560
ccgcgcacgcg	gccccgtcac	tctggactct	tttttttttt	ttttaaatttc	ttttaaatttc	19620
tggAACCTTA	aggtcgacca	gttgtccgtc	tttactcat	tcataatagg	cgaccgggtgg	19680
tactttgtct	ttttctgaaa	atcgcgagg	tcgaccagat	gtcagaaagt	ctgggtgtcg	19740
ataaaattatc	tgatctagat	ttgtttttct	gtttttcgt	tttgcgttgc	tttgcgttgc	19800
tttgcgttgc	tttgcgttgc	ttttttttgt	tttgcgttgc	tttgcgttgc	tttgcgttgc	19860
tttgcgttgc	tttgcgttgc	tttgcgttgc	tttgcgttgc	tttgcgttgc	tttgcgttgc	19920
tttgcgttgc	tttgcgttgc	tttgcgttgc	tttgcgttgc	tttgcgttgc	tttgcgttgc	19980
tttgcgttgc	tttgcgttgc	tttgcgttgc	tttgcgttgc	tttgcgttgc	tttgcgttgc	20040
tttgcgttgc	tttgcgttgc	tttgcgttgc	tttgcgttgc	tttgcgttgc	tttgcgttgc	20100
tacacaaaca	tgcactttt	ttaaaataaa	tttttaaaa	aaatgcgaaa	atcgaccaat	20160
tatcccttc	tttctcttc	tttttaaaa	atttcttt	tgtgtgtgt	tgtgtgtgt	20220
tgtgtgtgt	tgcgtgtgt	tgtgtgtgt	tgtgtgtgt	cgtcgacgt	cgccgcgc	20280
tacttataat	aatagggtcg	cgggtgggg	tagttcccg	gactccagag	gcagaggcag	20340
gcagacttct	gagttcgagg	ccagcctgg	ctacagagga	accctgtctc	gaaaaatgaa	20400
aataaaataca	tacatacata	catacataca	tacatacata	catacataca	tacatatgag	20460
gttgaccagt	tgtcaatcc	ttagaatttt	gttttaatt	aatgtatag	agagatagat	20520
aatagataga	tggatagagt	gatacaaata	tagtttttt	tttcagtaaa	tatgagggtt	20580
attaaccact	tttccctttt	tagtttttt	ttttttcc	ctgtccatgt	ggttgcgtgg	20640
atttgaactc	aggaccctgg	caggtcaact	ggaaaacgtg	ttttctatat	atataaata	20700
tggtctgtct	gtgtttttgt	ttttttgttgc	tttgcgttgc	tgttgcgttgc	cttgcgttgc	20760
tgcttttttt	tttctctcga	gacagtattt	ctctgtgtaa	cctgggtccc	tggaaactcac	20820
tctgttagacc	agcctggcct	caatcgact	cagaaatcc	cctgcctt	gtctaccc	

caattttgga	gtaaagggtgt	gctacaccac	tgcctggcat	tattatcatt	atcatttatta	20880
attttattat	tagacagaac	gaaatcaact	agttggtcct	gtttcgtaa	ttcatttgaa	20940
attagttgga	ccaatttagtt	ggctggggtt	ggaggtttct	tttggttccg	atttgggtgt	21000
ttgtggggct	ggggatcagg	tatctcaacg	gaatgcatga	aggttaaggt	gagatggctc	21060
gattttgtt	aagattactt	ttcttagtct	gaggaaaaaa	taaaataata	ttgggctacg	21120
tttcattgtc	tcatttctat	ttctctttct	ttcttcttct	ctttcagata	aggaggctgg	21180
ccagttccctc	ctgccttcgt	gaagatgtag	gcattgcatt	gggaaaagca	ttgtttgaga	21240
gatgtgtctag	tgaaccagag	agtttggatg	tcaagccgt	taatgtttat	tacaatata	21300
aaaagtctta	acaaagtgtat	ctttaactt	ttttttttt	tttctccccc	tacttctact	21360
tgttctca	ctgccaccaa	cgcgtttgt	acattgaatg	tgagcttgt	tttgccta	21420
agacatata	tttttctttt	ggttttgctt	gacatggttt	ccctttctat	ccgtgcaggg	21480
ttcccagacg	gccttttgag	aataaaatgg	gaggccagaa	ccaaagtctt	ttgaataaaag	21540
caccacaact	ctaaccctgtt	tggtgtttt	cctttccaa	gcacagatct	ttcccagcat	21600
ggaaaagcat	gtacgttgt	tagacacac	tagacgagag	caccagatct	cattgtgggt	21660
ggttgtgaac	caccaccaat	gtgggtgc	gggatttga	ctcaggatct	tcagaagacg	21720
agtcagggt	ctaaaccgt	gagccatctc	tccagccctc	ctacattct	tcttaaggca	21780
tgaatgatcc	cagcatggg	agacagtc	ccctttgt	ggtatatcac	catatactca	21840
ataaaataat	gaaatgaatg	aagtctccac	gtattttttt	tttcgagcta	tctaaattct	21900
ctcacagcac	ctccccctcc	cccacactgc	ctttctccct	atgtttgggt	ggggctgggg	21960
gagggggtgg	gtggggcag	ggatctgc	gtctcttgc	aggctgtga	actatttgc	22020
atggcctgt	tctctgaact	gttgagcc	gtctatccag	aggctgactg	gctagtttc	22080
tacctgaagt	ccctgagtga	tgatttccct	gtgaattc			22118

&lt;210&gt; 6

&lt;211&gt; 175

&lt;212&gt; DNA

&lt;213&gt; Mus Musculus

<400> 6						
ctcccgcccg	gccccgtgt	tcgcccgtcc	cgtggcgcgg	acaatgcgg	tgtgcgtcca	60
cgtgtgcgtg	tccgtgcagt	gccgttgcgg	agtgccctgc	tctcctccctc	ctccccggca	120
cggttccac	ggttggggac	caccgtgac	ctgcctct	tcgggcctgg	atccg	175

&lt;210&gt; 7

&lt;211&gt; 755

&lt;212&gt; DNA

&lt;213&gt; Mus musculus

<400> 7						
ggtctgggtt	gaattgttga	cctcgctctc	gggtgcggcc	tttggggaa	ggcggggtcg	60
gtcgtgccc	gcccggacg	tgtgtcgccc	cccaacttccc	gtcgagggt	ggcggtggcg	120
gcggcggtt	tagtctcccg	tgttgcgtct	tcccggtctc	ttgggggggg	tgccgtcg	180
ttcggggccg	gcgttgttgc	gtttacgcag	gcttgggttgc	ggactgcctc	aggagtcgtg	240
ggcggtgt	ttcccgccgg	ttttgcctcg	cgtctgcctg	ctttgcctcg	ggtttgcttgc	300
gttcgtgtct	cgggagcggt	ggttttttt	tttttcgggt	cccggggaga	ggggtttttc	360
cgggggacgt	tcccgtcgcc	ccctgcggcc	ggttgggttt	cgtttgcggc	tgtgttcgtt	420
tccccttccc	cgttgcggc	tgggttc	ccgtcggtc	ggccctctcc	ccggcggtc	480
gcccggccgt	gctgcccggac	ccccctct	ggggggatg	cccgcccacg	cacgcgtccg	540
ggcgccact	gtggtccggg	agctgcgtcg	caggcggtg	agccagttgg	agggcggtca	600
tggcccccgg	ggctcccgtg	gcccacgcgg	cgttcttct	gggggggcct	gtgcgtcg	660
gaaggctgcg	cacgtgtcg	gtcctgcga	ggaaagagg	ctttttttt	ttaggggtc	720
gtccttcgtc	gtcccggtcg	cggtggatcc	ggct			755

&lt;210&gt; 8

&lt;211&gt; 463

&lt;212&gt; DNA

&lt;213&gt; Mus musculus

<400> 8						
ggccgagggtg	cgtctgcggg	ttggggctcg	tccggccccc	tgcgcctccg	ggaaggcg	60

tagcgggtac	cgtcgccgcg	ccgagggtggg	cgcacgtcgg	ttagataacc	ccgagcgtgt	120
ttctgggtgt	tggcggcggg	ggctccggc	gatgtttcc	cctccccctc	tcccccgggc	180
caggtcagcc	tccgcctgtg	ggcttcgtcg	gccgtctccc	ccccoactac	gtccctcgcg	240
agcgagcccg	tccgttcgac	tttccttcg	cctttccccc	atcttccgc	gtccgttgg	300
ccccgggggtt	ttcacggcgc	cccccacgt	cctccgcctc	tccggccgtg	gtttggacgc	360
ctggttccgg	tctccccgca	aaaccccggt	tgggttggtc	tccggccccg	gcttgctt	420
cgggtctccc	aaccccgca	cggaagggtt	cgggggttcc	ggg		463

<210> 9  
<211> 378  
<212> DNA  
<213> Mus musculus

<400> 9						
ggattctca	ggattgaaac	ccaaaccgg	tca	ttccggctcc	ggccgggggg	60
ggcggccccc	ggcgggttgg	tgagtttagat	aac	cgatcgac	ccccccgtgg	120
ccggcgtc	ccattcgaac	gtctggcc	tcaactt	atggtagt	atgtgcctac	180
catggtgacc	acgggtgacg	ggaatcagg	gtttagattc	ggagaggag	cctgagaaac	240
ggctaccaca	tccaaggaag	gcagcaggcg	cgcaaaattac	ccactccca	ccccgggagg	300
tagtgacgaa	aaataacaat	acaggactt	ttcgaggccc	tgttaattt	atgagtccac	360
ttaaatcct	ttaagcag					378

<210> 10  
<211> 378  
<212> DNA  
<213> Mus musculus

<400> 10						
gatccattgg	agggcaagtc	tggtgc	agccgcggta	attccagctc	caatagcgta	60
tattaaagt	gctcagtt	aaaagctcg	atggatct	tgggagcggg	cggcgggtcc	120
gcccgcggc	gagtcaccgc	ccgtccccgc	cccttgc	tcggcgc	ctcgatgtc	180
ttagctgagt	tgtcccg	ggcccgaa	gtttactt	aaaaaattag	agttgttca	240
aagcaggccc	gagccgc	gataccgca	gatggaaat	aatggatag	gaccgcgg	300
cctatttgt	ttgggtt	gaactgagcc	catgattaag	ggaaacggcc	gggggcattc	360
ccttattgc	ccccctca					378

<210> 11  
<211> 719  
<212> DNA  
<213> Mus musculus

<400> 11						
ggatcttcc	cgctccccgt	tcctccggc	ccctccaccc	gcgcgtctcc	cccccttctt	60
tcccctctcc	ggaggggggg	gagggtgggg	cgcg	gggtcg	tgggtcg	120
gggggaccgc	ccccggccgg	caaaaggccg	ccggcggcg	cacttcaacc	gtacgggt	180
gcccgcaccg	gtacgagac	ggctggaa	gcccgcagg	aatgtggct	cggggggg	240
ggcgcgtc	agggcgcc	gaaccac	accccgag	ttacagcc	ccggccgc	300
tttcgcggaa	tcccg	gaggggaa	ccgataaccc	tcggcg	tttccc	360
ccccgtc	ctccggcg	ggcg	tggggccgg	gccgc	ccacgccc	420
ggtttcttc	tcccccgtc	tcggccgtt	tgggggggg	agcccgtt	ggggcg	480
ggactgtct	cagtgcgc	cgggcg	cg	ggcccgg	gttctct	540
tcacgcgc	cccgacga	ccgagc	gggtcg	cgatgt	ctacccac	600
gaccgcgtt	gaaacacgg	ccaagg	taacgcgt	cgagtc	ggctcg	660
aaagccccc	tggcgaat	agg	cccg	ggggcccg	gtggat	719

<210> 12  
<211> 685  
<212> DNA  
<213> Mus musculus

<400> 12  
 cgaggcctct ccagtccgcc gagggcgac caccggccc tctcgcccc cgcgtcgggg 60  
 aggtggagca cgagcgtacg cgtaggacc cgaaagatgg tgaactatgc ctggcaggg 120  
 cgaagccaga ggaaactctg gtggagggtcc gtagcggtcc tgacgtgoaa atcggtcg 180  
 cgacctgggt atagggcga aagactaatac gaaccatcta gtagctgggt ccctccgaag 240  
 tttccctca gatactggc gctctcgcaa ccttcggaaag cagtttatac cggtaaagg 300  
 cggaatggat taggaggtct tggggccgga aacgatctca aactatttct caaaactttaa 360  
 atgggttaagg aagcccggt cgctggcgtg gagccggcg tggatgcga gtgcctagtg 420  
 ggccactttt ggtaaagcaga actggcgctg cggatgaac cgaacgcgg gtttaaggcgc 480  
 cccatcgcccgatcgatccatcg accccagaaa aggttgttgg ttagatagac agcaggacgg 540  
 tggccatggatcgatccatcgatccatcg accccagaaa aggttgttgg ttagatagac agcaggacgg 600  
 gcccgtccatcgatccatcg accccagaaa aggttgttgg ttagatagac agcaggacgg 660  
 aacgggacgg gacggagcg gcccgc 685

<210> 13  
 <211> 5162  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Chimeric bacterial plasmid

<400> 13  
 gacggatcg gagatctccc gatcccatt ggtcgactct cagtacaatc tgctctgatg 60  
 ccgcatagtt aagccagtat ctgctccctg ctttgtgttt ggaggtcgct gagtagtgc 120  
 cgagcaaat ttaagctaca acaaggcaag gcttggaccga caattgcatt aagaatctgc 180  
 tttaggttag gcttttgcg ctgcttcgcg atgtacggc cagatatacg cgttgacatt 240  
 gattatttgcg tagtattaa tagtaatcaa ttacgggttc attagttcat agccatata 300  
 tggagttccg cgttacataa ttacggtaa atggccgc tggctgaccg cccaaacgacc 360  
 cccgcccatt gacgtcaata atgacgtatg ttccatagt aacgccaata gggactttcc 420  
 attgacgtca atgggtggac tatttacgtt aaactgccc cttggcagta catcaagtgt 480  
 atcatatgcc aagtacgccc cctattgcac tcaatgacgg taaatggccc gcctggcatt 540  
 atgcccagta catgacccat tgggactttc ctacttggca gtacatctac gtattgtca 600  
 tcgcttattac catggtgatg cggttttgc agtacatcaa tggcgttgc tagcggtttgc 660  
 actcacggg atttcaagt ctccacccca ttgacgtcaa tggaggtttg tttggcacc 720  
 aaaatcaacg ggactttcca aaatgtcgta acaactccgc cccattgcac caaatggcgc 780  
 gttaggcgtgt acgggtggag gtctatataa gcagagctct ctggctactt agagaaccca 840  
 ctgcttactg gcttatcgaa attaatacgat ctcactatag ggagacccaa gcttggtacc 900  
 gagctcgat cgatatctgc ggccgcgtcg acgaaatca gtggatccac tagtaacggc 960  
 cgccagtgtg ctggattaa ttgcgtgtct gcgaggggca gcttgggggg tgtagtactcc 1020  
 ctctcaaaag cgggcatgac ttctgcgtca agattgtcag tttccaaaaaa cgaggaggat 1080  
 ttgatattca cctggcccgc ggtgatgcct ttgagggtgg cccgcgtccat ctggcagaa 1140  
 aagacaatct ttttgtgtc aagcttgagg tggcgttgc ttgagatctg gccatacact 1200  
 tgagtgacaa tgacatccac ttgcctttc tctccacagg tggccactcc cagggtccaa 1260  
 tgcaggtcga gcatgtatct agggcgccca attccgcccc tctccctccc cccccccctaa 1320  
 cgttactggc cgaagccgct tggaaataagg cccgtgtgcg tttgtctata tggatatttc 1380  
 caccatcc cccgttttgc gcaatgtgag ggcccgaaa cctggccctg tcttcttgac 1440  
 gagcattctt aggggtctt cccctctcgca caaaggaatg caaggtctgt tgaatgtcg 1500  
 gaaggaagca gttctctgg aagcttctt aagacaaaca acgtctgtag cggcccttt 1560  
 caggcaggg aaccccccac ctggcgacag gtgcctctgc ggccaaaagc cacgtgtata 1620  
 agatcacatct gcaaaaggcg cacaacccca gtgccacgtt gtgagttgg tagttgtgg 1680  
 aagagtcaaa tggctctctt caagcgtatt caacaagggg ctgaaggatg cccagaagg 1740  
 accccatgtt atggatctg atctggggcc tgggtgcaca tgcttacat gtgttagtc 1800  
 gaggttaaaa aaacgtctag gccccccgaa ccacggggac gtggtttcc ttgaaaaac 1860  
 acgtatgtt gcttgcaca acccggtatc caccggtcg caccatgtt agcaaggcg 1920  
 aggagctttt caccgggtt gtgcctatcc tggtcgagct ggacggcgac gtaaacggcc 1980  
 acaagttcag cgtgtccggc gagggcgagg gcgttgcac ctacggcaag ctgaccctga 2040  
 agttcatctg caccacccgc aagctggcc tggccctggcc caccctcgat accaccctga 2100  
 cctacggcgt gcaatgttcc agccgttacc cccgaccat gaagcagcgc gacttcttca 2160  
 agtccgcatt gcccgaaggc tacgtccagg agcgcaccat cttcttcaag gacgacggca 2220

actacaagac	ccgcggccgag	gtgaaggttcg	agggcgacac	cctggtaac	cgcatcgagc	2280
tgaaggccat	cgacttcaag	gaggacggca	acatctggg	gcacaagctg	gagtacaact	2340
acaacagcca	caacgtctat	atcatggccg	acaagcagaa	gaacggcattc	aaggtaact	2400
tcaagatccg	ccacaacatc	gaggacggca	gctgtcagct	cgccgaccac	taccagcaga	2460
acaccccccatt	cgcgacggc	cccgtctgc	tgcccgacaa	ccactacctg	agcacccagt	2520
ccgcctgag	caaagacccc	aacgagaagc	gcgatcacat	gttcctgctg	gagttcgtga	2580
ccgcccgg	gatcactctc	ggcatgacg	agctgtacaa	gtaaagcggc	cctagagctc	2640
gctgatcagc	ctcgactgtg	cctctagtt	ccagccatct	gttgttgc	cctccccgt	2700
gccttcctt	acccttggaa	gtgccactcc	cactgtcctt	tcctaataaa	atgaggaaat	2760
tgcacatcgat	tgtctgagta	ggtgtcattc	tattctgggg	ggtgggggtgg	ggcaggacag	2820
caagggggag	gatttggaa	acaatagcag	goatgtggg	gatgcgggtgg	gtcttatggc	2880
ttctgaggcg	gaaaagaacca	gctggggctc	gagtgcattc	tagttgtgt	ttgtccaaac	2940
tcatcaatgt	atcttatcat	gtctgtatac	cgtcgaccc	tagctagagc	ttggcgtaat	3000
catggtcata	gctgtttcct	gtgtgaaatt	gttatccgc	cacaattcca	cacaacatac	3060
gagccggaag	cataaaagtgt	aaagctggg	gtgcctaattg	agttagctaa	ctcacattaa	3120
ttgcgttgcg	ctcaactgccc	gctttccagt	cgggaaacct	gtcgtgccag	ctgcattaat	3180
gaatcgccca	acgcgcgggg	agaggcggtt	tgctgtattgg	gchgcttcc	gcttcctcgc	3240
tcactgactc	gctgcgctcg	gtcgttgcgc	tgccggcagc	gttacatcgct	cactcaaagg	3300
cggttaatacg	gttatccaca	gaatcagggg	ataacgcagg	aaagaaacatg	tgagcaaaag	3360
gccagcaaaa	ggccaggaac	cgtaaaaagg	ccgcgttgc	ggcgttttc	cataggctcc	3420
gccccctgaa	cgagcatcac	aaaaatcgac	gctcaagtca	gagggtggcga	accccgacag	3480
gactataaag	ataccaggcg	tttccccctg	gaagctccct	cgtgcgtct	cctgttccga	3540
ccctgcgcgt	taccggatac	ctgtccgcct	ttctcccttc	ggaaagcggtg	gchgcttcc	3600
aatgctcagc	ctgttaggtat	ctcagttcg	tgttagtgcgt	tcgctccaag	ctgggctgtg	3660
tgcacgaacc	ccccgttca	ccgcaccgc	gchccttatac	cggtactat	ctgtttgtat	3720
ccaaacccgt	aagacacgcac	ttatcgccac	ttggcagcagc	cactggtaac	aggatttagca	3780
gagcgaggta	tgttagcggt	gctacagagt	tcttgaagtg	gtggcctaacc	tacggctaca	3840
ctagaaggac	agatatttgg	atctgcgctc	tgctgaagcc	agttaccc	ggaaaaaagag	3900
tttgttagtct	ttgatccggc	aaacaaacca	ccgtgttag	cggtgtttt	tttgtttgca	3960
agcagcagat	tacgcgcaga	aaaaaaggat	ctcaagaaga	tcctttgatc	ttttctacgg	4020
ggtctgacgc	tcagttggaa	aaaaactcac	gtttagggat	tttggtcatg	agattatcaa	4080
aaaggatctt	caccttagatc	cttttaaaat	aaaaatgaag	ttttaaatca	atctaaagta	4140
tatatgagta	aacttggtct	gacaggattac	aatgtttat	cagtggacca	cctatctcag	4200
cgatctgtct	atttcgttca	tccatagtt	cctgactccc	cgtcggttag	ataactacga	4260
tacgggaggg	cttaccatct	ggccccagtg	ctgcaatgtat	accgcgagac	ccacgctcac	4320
cggctccaga	tttatcagca	ataaaccagc	cagccggaa	ggccgagcgc	agaagtggtc	4380
ctgcaactt	atccgcctcc	atccagttca	ttaattgtt	ccgggaagct	agagtaagta	4440
gttcggcagt	taatagttt	cgcaacgtt	ttgccattgc	tacaggcatc	gtgggtgtc	4500
gctcgctgtt	tgttatggct	tcatttcagct	ccgggtccca	acgatcaagg	cgagttacat	4560
gatccccat	gttggcataaa	aaagcggtt	gctccttcgg	tcctccgatc	gttgcagaa	4620
gtaaggttgc	cgcgtgtt	tcactcatgg	ttatggcagc	actgcataat	tctcttactg	4680
tcatgccatc	cgtaaagatgc	ttttctgtga	ctgggtgagta	ctcaaccaag	tcattctgag	4740
aatagtgtat	gcggcgaccg	agttgtctt	gcccggcgtc	aatacggat	aataccgcgc	4800
cacatagcag	aactttaaa	gtgctcatca	ttggaaaacg	ttcttcgggg	cgaaaactct	4860
caaggatctt	accgctgtt	agatccagg	cgatgttaacc	cactcgtgc	cccaactgat	4920
cttcagcatc	ttttacttt	accagcgtt	ctgggtgagc	aaaaacagga	aggcaaaatg	4980
ccgaaaaaaaa	gggataagg	gacacacgg	aatgttgaat	actcatactc	ttccttttc	5040
aatattattt	aagcatttat	cagggttatt	gtctcatgag	cggtatcata	tttgaatgt	5100
tttagaaaaa	taaacaata	gggggtccgc	gcacattcc	ccgaaaagt	ccacctgacg	5160
tc						5162

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization International Bureau



(43) International Publication Date  
13 November 2003 (13.11.2003)

PCT

(10) International Publication Number  
**WO 2003/093469 A3**

(51) International Patent Classification<sup>7</sup>: C12N 15/64, 15/88, A61K 48/00

(74) Agent: SMART & BIGGAR; Box 11560, Vancouver Centre, Suite 2200, 650 West Georgia Street, Vancouver, British Columbia V6B 4N8 (CA).

(21) International Application Number:  
PCT/CA2003/000645

(81) Designated States (*national*): AE, AG, AL, AM, AT (utility model), AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ (utility model), CZ, DE (utility model), DE, DK (utility model), DK, DM, DZ, EC, EE (utility model), EE, ES, FI (utility model), FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK (utility model), SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(22) International Filing Date: 30 April 2003 (30.04.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
60/377,547 1 May 2002 (01.05.2002) US

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

(71) Applicant (*for all designated States except US*): CHROMOS MOLECULAR SYSTEMS, INC. [CA/CA]; 8081 Lougheed Highway, Burnaby, British Columbia V5A 1W9 (CA).

Published:

— with international search report

(88) Date of publication of the international search report:  
8 April 2004

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.



A3

**WO 2003/093469 A3**

(54) Title: METHODS FOR DELIVERING NUCLEIC ACID MOLECULES INTO CELLS AND ASSESSMENT THEREOF

(57) Abstract: Methods for delivering nucleic acid molecules into cells and methods for measuring nucleic acid delivery into cells and the expression of the nucleic acids are provided. The methods are designed for introduction of large nucleic acid molecules, including artificial chromosomes, into cells.

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/CA 03/00645

**A. CLASSIFICATION OF SUBJECT MATTER**  
 IPC 7 C12N15/64 C12N15/88 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
 IPC 7 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, WPI Data, MEDLINE, EMBASE, PAJ

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>WO 99 54445 A (INEX PHARMACEUTICALS CORP          ;GRAHAM ROGER W (CA); JOSHI PHALGUN B (C)          28 October 1999 (1999-10-28)</p> <p>page 4          page 10-12          page 17, line 26 - line 30          page 24; table 1          page 25, line 20 - line 31          page 26-29          page 35; example 1</p> <p>----</p> <p style="text-align: center;">-/-</p>	1-82

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

\* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 November 2003	05/01/2004
Name and mailing address of the ISA  European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer  Grötzinger, T

## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/CA 03/00645

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TSENG W-C ET AL: "Mitosis enhances transgene expression of plasmid delivered by cationic liposomes" BIOCHIMICA ET BIOPHYSICA ACTA. GENE STRUCTURE AND EXPRESSION, ELSEVIER, AMSTERDAM, NL, vol. 1445, no. 1, 14 April 1999 (1999-04-14), pages 53-64, XP004275361 ISSN: 0167-4781 abstract page 53, right-hand column, paragraph 1 page 54 page 56 -----	1-82
X	DE JONG G ET AL: "Efficient in-vitro transfer of a 60-Mb mammalian artificial chromosome into murine and hamster cells using cationic lipids and dendrimers" CHROMOSOME RESEARCH, vol. 9, no. 6, 2001, pages 475-485, XP009020460 ISSN: 0967-3849 abstract page 476, left-hand column, paragraph 1 page 477, left-hand column, paragraph 2 -----	1-82
X	RECH E L ET AL: "INTRODUCTION OF YEAST ARTIFICIAL CHROMOSOME VECTOR INTO SACCHAROMYCES-CEREVISIAE CELLS BY ELECTROPORATION" NUCLEIC ACIDS RESEARCH, vol. 18, no. 5, 1990, page 1313 XP001155836 ISSN: 0305-1048 the whole document -----	1-82
X	LEE JEANNIE T ET AL: "A method for high efficiency YAC lipofection into murine embryonic stem cells" NUCLEIC ACIDS RESEARCH, vol. 24, no. 24, 1996, pages 5054-5055, XP002260393 ISSN: 0305-1048 abstract page 5054, left-hand column, paragraphs 1,2 page 5055, left-hand column, paragraphs 2,4 -----	1-82

# INTERNATIONAL SEARCH REPORT

ation on patent family members

International Application No

PCT/CA 03/00645

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO 9954445	A 28-10-1999	AU	762986 B2	10-07-2003
		AU	3591299 A	08-11-1999
		AU	754244 B2	07-11-2002
		AU	3591399 A	08-11-1999
		CA	2325561 A1	28-10-1999
		CA	2325596 A1	28-10-1999
		WO	9954445 A2	28-10-1999
		WO	9954444 A2	28-10-1999
		EP	1082419 A2	14-03-2001
		EP	1082418 A2	14-03-2001
		JP	2002512257 T	23-04-2002
		JP	2002512258 T	23-04-2002